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HISTOPATHOLOGIC STUDY OF THE MODE OF INHIBITION OF CELLULAR PROLIFERATION BY URETHANE: EFFECT OF URETHANE ON WOUND HEALING[1,2]

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INTRODUCTION

Little is known of the manner in which urethane (ethyl carbamate) affects cellular proliferation. The use of urethane in the treatment of leukemia (*1–4*), metastatic anaplastic carcinoma (*5*), and prostatic carcinoma (*6*) has resulted in temporary remissions. These clinical effects indicate that if compounds of similar but greater effectiveness can be found, chemotherapy of inoperable cancer may be feasible. The search for such compounds would be aided by an understanding of the mode of action of urethane.

Although this drug is reputedly one of the most effective inhibitors of nuclear division, the first study of its effect on animal tumors (*7*) indicated that its alteration of the growth rate depended on more than a colchicinelike retardation of the mitotic cycle. In that study, the characteristic cellular structure of Walker rat carcinoma 256 was replaced by a fibrous tissue rich in stroma, a change seemingly toward increased differentiation according to Haddow. In the course of clinical remissions following urethane therapy in myelogenous leukemia (*3*), "differentiation of the circulating leukocytes toward more mature types" was observed as the total leukocyte count fell. The decrease in serum acid phosphatase observed in prostatic cancer during therapy with urethane was shown not to be the result of an antiandrogenic or estrogenic action (*6*). From these observations, it would seem possible that one of the ways in which urethane retards tumor growth is by altering the growth characteristics of rapidly proliferating cells so that differentiation can occur.

In order to elucidate the mode of action of urethane, its effect on wound healing was studied. In the normal healing process, fibroblasts and new capillaries are produced which under the influence of normal growth-regulating substances proliferate more rapidly than many neoplastic cells and finally mature and form collagenous connective tissue.

EXPERIMENTAL PROCEDURE

Two hundred albino rats were used in these experiments: 170 of them were normal, averaging 215 gm. in weight, and 30 were protein-depleted, averaging 150 gm. With effective doses of urethane, it became apparent at once that the severe weight loss was an important modifying factor necessitating adequate controls. For this purpose, normal controls were starved throughout the experimental period, and other rats (protein-depleted) previously rendered hypoproteinemic and emaciated by prolonged feeding of a synthetic diet containing 1.9 percent of protein (*8*) were used in order to determine whether the effect of inanition augmented the action of urethane.

All injections were made intraperitoneally. In preliminary experiments, 200 and 500 mg. of urethane per kilogram daily did not seem to affect wound healing. Therefore, the largest amount of urethane that killed the least rats in successive days of treatment was used in the final experi-

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[2] Part of the work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army, and the University of Chicago Toxicity Laboratory. Under the terms of the contract, the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

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ments. This proved to be 1,000 mg. per kilogram, in two equal doses at least 8 hours apart. A single daily dose of 1,000 mg. per kilogram killed 60 percent of the rats in 3 days. Since the protein-depleted rats could not tolerate urethane much in excess of 200 mg. per kilogram daily, this dose was used with these animals and was given to a comparable group of normal control rats.

Urethane is a bacteriostatic agent in addition to its other actions (*9*). Since it might thereby modify wound healing by preventing infection, this action was controlled by giving the control animals urea in doses comparable with the doses of urethane. Urea, while chemically related to urethane, is relatively nontoxic but similarly bacteriostatic. Other rats received comparable amounts of distilled water intraperitoneally.

The wounds, made as closely alike as possible, were right para-median laparotomy incisions 3 cm. in length. They were immediately closed by three evenly placed, stainless-steel-wire sutures.

Two types of experiments were performed. In one the wounds were made after the animals had received the intraperitoneal injection daily for 4 days. In the other, the drugs were given for the first time 48 hours postoperatively. The pertinent data are given in table 1. In the first type of experiment, studies were made of the effect on wound healing of the following, administered daily both preoperatively and postoperatively: distilled water (group 1a); 200 mg. of urea per kilogram in normal (group 1b) and in protein-depleted rats (2a); 200 mg. of urethane per kilogram in normal (1d) and in protein-depleted (2b) rats; 1,000 mg. of urea per kilogram plus starvation in normal rats (1c) and 1,000 mg. of urethane per kilogram in normal rats (1e). In the second, studies were made of the effects on growing fibroblasts and capillaries, of a single administration of 1,000 mg. of urethane or urea per kilogram and the daily administration of this amount in divided doses, in normal rats (table 1, groups 3a and 3b).

Postoperatively, in all experiments, the animals were killed, in each group, at daily intervals for 5 days. Paraffin sections stained with hematoxylin and eosin were made from the central portion of each wound. Representative sections were stained for collagen and reticulum.

TABLE 1.—*Effect of urethane on the proliferation and mitoses of fibroblasts and endothelial cells in the healing of surgical incisions in rats*

[Numerator, Average total number of mitoses in these areas. Denominator, Average total number of fibroblasts and endothelial cells in these areas]

Group[1]	Number of rats	Hours postoperative										Final state of wound
		24		48		72		96		120		
		Field[2]		Field[2]		Field[2]		Field[2]		Field[2]		
		A	B	A	B	A	B	A	B	A	B	
1a	11	0/10	0/87	0/147	1/130	2/209	1/181	1/243	0/185	2/387	1/127	Healed.
1b	20	0/27	0/64	0/83	0/95	0/158	5/226	6/328	2/304	1/310	4/280	Do.
1c	10	1/103	0/59	1/163	0/80	1/150	0/165	6/478	0/192	4/311	2/261	Do.
1d	33	0/61	0/100	0/121	1/115	1/159	4/174	7/367	5/233	4/361	4/246	Do.
1e	16	0/25	0/40	1/117	0/40	0/63	1/119	0/91	1/124	1/192	0/105	Open.
2a	12	0/23	0/40	0/35	1/128	0/202	1/237	0/262	3/393	3/291	5/525	Healed.
2b	13	0/50	0/26	0/70	0/65	0/114	0/105	0/72	0/113	0/89	0/144	Open.
3a	15	.....	.....	1/101	3/259	1/134	1/199	1/214	1/360	.....	.....	Healed.
3b	11	.....	.....	.....	.....	1/85	0/170	0/78	0/148	.....	.....	Open.

[1] Groups 1a-1e received a daily intraperitoneal administration preoperatively and postoperatively of: 1a, 1.0 ml. distilled water; 1b, 200 mg. urea per kilogram; 1c, 1,000 mg. urea per kilogram plus starvation; 1d, 200 mg. urethane per kilogram; and 1e, 1,000 mg. urethane per kilogram. Groups 2a and 2b (protein-depleted rats) received a daily intraperitoneal administration of 200 mg. of urea and 200 mg. of urethane per kilogram, respectively. Groups 3a and 3b received 1,000 mg. of urea and urethane per kilogram, respectively, starting 48 hours postoperatively.

[2] A is the average total number of these cells and mitoses in a field the length of the wound and the width of one high-power microscopic field (×450); B is the average total number of these cells and mitoses in 2 high-power fields the length of the wound and 1.0 mm. distant from the wound on each side.

RESULTS

Wound Healing in Normal Rats

During the first 24 hours, the wounds of the rats in group 1a that received 1.0 ml. of distilled water preoperatively and postoperatively became edematous and developed an exudate of polymorphonuclear leukocytes and fibrin. By 48 hours, fibroblastic and endothelial proliferation was well under way in the perivascular connective tissue near the wound. The exudate then consisted chiefly of lymphocytes and macrophages. By 72 hours, the new fibroblasts and endothelial

cells were growing across the incision. By 96 hours, most of the wounds were completely grown together. The connective tissue was differentiating as evidenced by prominent formation of capillaries and venules, increase in amount of interstitial substance, and a decrease in the number of mitoses and young fibroblasts. By 122 hours, the final observation period, all these wounds were well-healed. Collagen was abundant. Most of the fibroblasts were reverted to the resting stage.

Except for a smaller number of polymorphonuclear leukocytes in the exudate after 24 hours, the wounds in the animals in group 1b receiving 200 mg. urea healed in a manner identical with that of the wounds in group 1a. By 96 hours, healing was almost complete (fig. 1, *A*).

The wounds of the animals in group 1c (1,000 mg. of urea per kilogram and starvation) healed at the same rate as did those of groups 1a and 1b.

The wounds of animals in group 1d (200 mg. of urethane per kilogram) were identical with those of the preceding group for the first 72 hours. By 96 hours, however, there was little evidence of organization in most of them. The region of the incision was virtually a tissue culture of proliferating fibroblasts. The number of mitotic figures in these cells exceeded that of any other group of animals at any stage (fig. 1, *B* and *F*). Capillary proliferation was overshadowed by fibroplasia at 96 hours. Collagen formation and capillary organization were progressing well at 122 hours but were not so advanced as in the preceding groups of animals.

The large dose of urethane (1,000 mg. per kilogram) administered to the animals of group 1e altered the healing processes throughout the 5-day observation period. By 24 hours, edema and fibrin were minimal in amount. Polymorphonuclear leukocytes were either absent or sparsely and focally distributed. By 48 hours, the wounds were edematous and contained fibrin. In the intervening 24 hours the polymorphonuclear leukocytic infiltration had reached normal proportions but had not been replaced by lymphocytes and macrophages as in the other groups. A small number of macrophages contained nuclear debris, but the exudate consisted chiefly of degenerate leukocytic forms and free nuclear fragments. New fibroblasts and capillary buds were not found until the 72-hour period. These were proliferating sparsely in the adventitial connective tissue far from the incision. Most of the fibroblasts appeared attenuated. By 96 hours, the focal areas of new fibroblasts were slightly more prominent but were so distant from the wound that the margins were unaffected (fig. 1, *C*). An occasional mitotic figure was found, but most of the fibroblasts were thin and adjacent to dense collagen bundles. Although capillary proliferation was meager, the new capillaries were organizing into venules. By 122 hours, these wounds were still open. The few areas of apparently good fibroblastic and capillary growth were not obviously larger than they were at 96 hours.

Wound Healing in the Protein-Depleted Rat

As has been previously shown (*10*), the process of wound healing in the protein-depleted rat was retarded by the poor nutritive state. Wound infection could not be entirely prevented. In the first 24 hours, the only difference in the wounds of the rats in group 2a that received 200 mg. of urea and the wounds in the first two groups of normal rats was an increase in muscle necrosis caused by an apparent increased susceptibility to trauma. Polymorphonuclear leukocytes were as numerous as in the normal animals. By 48 hours, fibroblastic and endothelial proliferation was under way but was not so extensive as in the normal animals. This reduction in quantity rather than in quality persisted throughout the observation period. After 96 hours, for example, while fibroblasts and endothelial cells were progressing across the wound, these cells were not so numerous as in the normal animals, more wandering lymphocytes and polyblasts were present than in the controls at this state, and collagen formation was poor (fig. 1, *D*).

In the protein-depleted rats that received 200 mg. of urethane per kilogram (group 2b), the process of wound healing was further reduced until it approximated that of the normal animals on 1,000 mg. of urethane per kilogram (group 1e). Cellular exudation in 24 hours was slight. Fibroblastic and endothelial proliferation was not in evidence until 72 hours. At this time, the fibroblasts were sparse and were apparently growing predominantly along the lines of stress rather than in the exuberant fashion seen in the controls. This phenomenon persisted through 96 hours (fig. 1, *G*). The exudate at 72 hours consisted chiefly

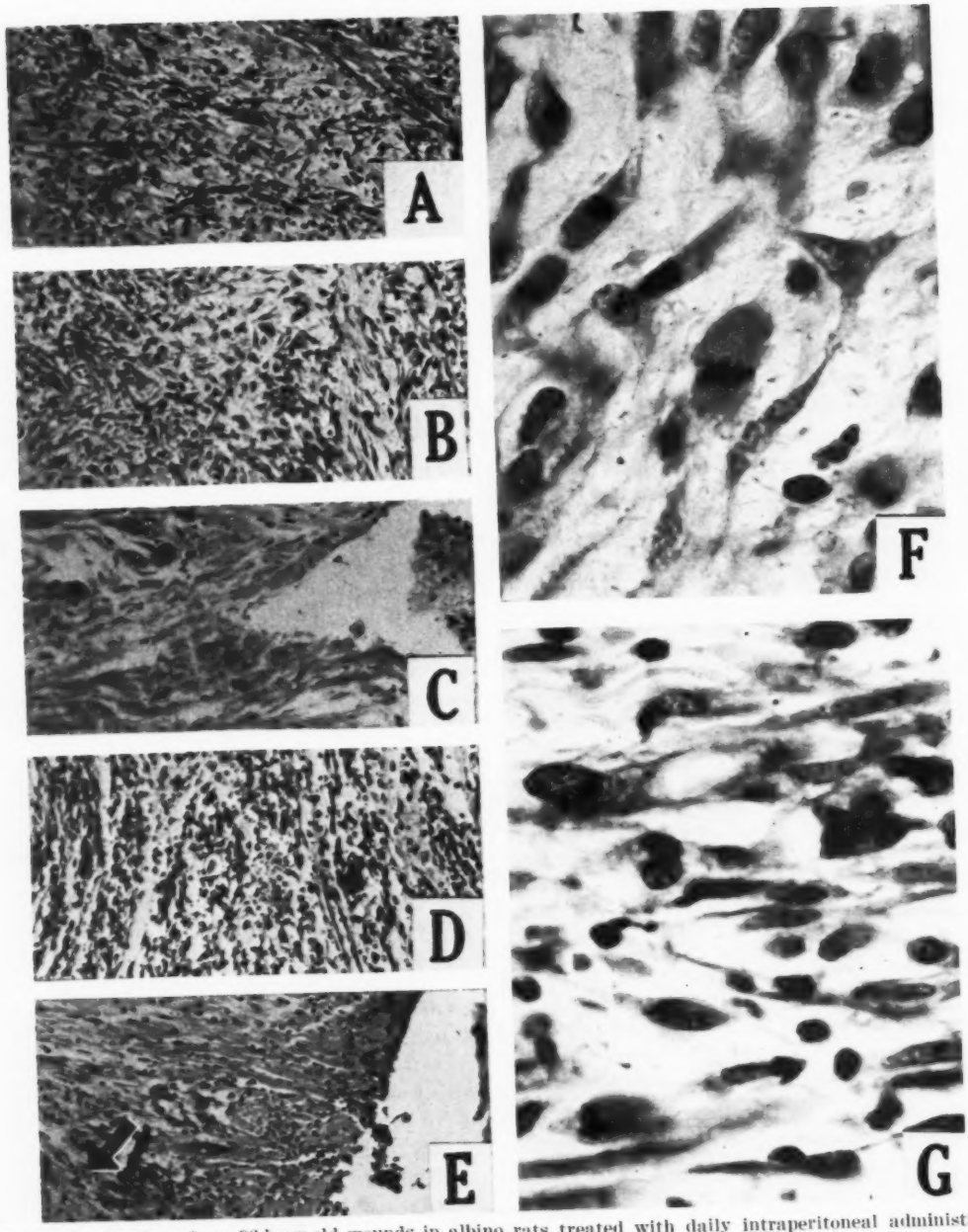

FIGURE 1.—Sections from 96-hour-old wounds in albino rats treated with daily intraperitoneal administration of various substances. *A*, From rat that received 200 mg. of urea per kilogram, showing well-advanced healing. × 125. *B*, From rat that received 200 mg. of urethane per kilogram, showing continued marked fibroblastic proliferation. × 125. *C*, From rat that received 1,000 mg. of urethane per kilogram, showing lack of healing near wound. × 125. *D*, From protein-depleted rat that received 200 mg. of urea per kilogram, showing slightly reduced healing. × 125. *E*, From protein-depleted rat that received 200 mg. of urethane per kilogram, showing lack of healing at wound and (arrow) area of most marked fibroplasia. × 125. *F*, Area in *B*, showing proliferating fibroblasts. × 1100. *G*, Marked area of fibroplasia in *E*, showing attenuated appearance of fibroblasts and endothelium. × 1100.

of polymorphonuclear leukocytes. In addition, so many macrophages and polyblasts were present that the question was raised whether the development of these cells into fibroblasts had been arrested. By 96 and 122 hours what fibroplasia and capillary formation there were had still failed to reach the actual wound site (fig. 1, *E*). In spite of this failure in several instances, capillary organization and collagen formation were occurring, but no further proliferation was evident.

Wound Healing in Normal Rat Following Daily Administration of 1,000 mg. of Urethane per Kilogram Started 48 Hours Postoperatively

Forty-eight hours postoperatively (the time when urethane was first given to animals of group 3b), fibroblasts were proliferating around the normal capillaries and arterioles, between the sarcolemmal sheaths, and in the preperitoneal areolar tissue adjacent to the wound. Fibroblastic and capillary proliferation continued throughout the observation period in the presence of 1,000 mg. of urethane per kilogram but so slowly that by 96 hours the new fibroblastic tissue and capillaries were not much more extensive than they were at 48 hours. The wounds were not healed by 120 hours, but in the areas of fibroplasia collagen had been produced, many of the fibroblasts seemed to be reverting to the resting stage, and capillary organization was evident.

Effect of Urethane on the Mitosis of Fibroblasts and Endothelium

The mitotic activity of the fibroblasts and endothelial cells was studied in the wounds of all these animals (table 1). The number of mitotic figures in these cells was slightly reduced in the untreated, protein-depleted rats and was greatly reduced in the protein-depleted animals by the 200 mg. of urethane per kilogram daily (fig. 1, *G*). A similar reduction was observed in normal animals treated with 1,000 mg. of urethane per kilogram daily. No abnormal mitotic figures were found under the influence of urethane. The number of mitotic figures in the fibroblasts was increased over that of the normal controls by the daily administration of 200 mg. of urethane per kilogram in the normal animals (fig. 1, *F*). After a single dose of 1,000 mg. of urethane per kilogram 48 hours postoperatively, there was no accumulation of mitotic figures as would have been expected if this substance had a colchicinelike effect on the nucleus (*11*). In fact, the number of mitotic figures in these animals was reduced.

DISCUSSION

It would seem from these experiments that urethane inhibits reparative cellular proliferation in the healing of a sterile surgical wound, predominantly indirectly. Cellular exudation is delayed. After exudation occurs, the removal of the debris of the degenerating leukocytes is also delayed. This delay apparently results from the failure of macrophages to appear or to develop in the wound as rapidly as in the normal animals. The greatest inhibition is seen among the fibroblasts and endothelial cells. These cells are delayed in appearance; once they develop, they fail to proliferate rapidly enough to reach and cross the wound. This inhibition is best seen when healing is allowed to proceed undisturbed for 48 hours and then put under the influence of urethane. Proliferation of these cells then becomes extremely slow, but differentiation, as gaged by collagen formation and capillary organization, proceeds at the normal rate. The fact that the formation of adult connective tissue was not found in any group earlier than 96 hours is evidence against urethane's being a stimulant to increased differentiation of connective-tissue cells. The appearance of the retarded fibroblasts, arranged along the lines of the previously existing framework, is more likely the result of a lack of growth energy rather than an early tendency toward differentiation. Likewise, in Haddow's experiments (*7*), the increase in fibrous stroma might be due to a condensation of previously existing connective tissue rather than the result of fibroblastic maturation. The increased number of mitotic figures in the luxuriant growth of fibroblasts after 96 hours of healing in the normal animals with 200 mg. of urethane per kilogram may be evidence that in small doses this drug is stimulative to fibroblastic proliferation.

The observation that mitotic figures do not accumulate or "freeze" in any one stage of mitosis in the presence of urethane confirms Haddow's observations (*7*) that this substance does not have an effect similar to that of colchicine. The absence of abnormal and degenerating mitotic figures or nuclear remnants in dead cells does not support the

theory that urethane has a direct toxic effect on the dividing nucleus.

While the dose of urethane required to inhibit mitosis reportedly varies considerably, 1,000 mg. per kilogram appears to be the amount that best inhibits rat and mouse corneal mitoses (*12*), reduces mitosis and causes remission in mouse myeloid leukemia (*13, 14*), inhibits the growth of transplantable tumors (*6, 15, 16*), and causes 50 to 60 percent decrease in circulating lymphocytes (*17*). This dose also caused a marked loss of body weight, which is generally considered a reliable indication of severe systemic intoxication. The uncompensated alkalosis in rabbits and rats treated with 1,000 mg. of urethane per kilogram (*17*), the deaths of rats following sublethal X-ray exposure after urethane anethesia (*17*), and the death of rabbits and men after combined sublethal doses of urethane and chloroform (*18*) are further indications of the systemic toxicity and the consequent reduced resistance to injury caused by this drug. The inhibition of fibroplasia by urethane may also belong in this category. In fact, these experiments question whether urethane acts primarily as a local growth inhibitor. The augmentation of the effectiveness of urethane by preliminary protein depletion was greater than could be expected from the moderate decrease in fibroplasia in the wounds of the untreated protein-depleted rats. This phonomenon suggests that urethane may stop cellular proliferation by so altering general metabolic processes that substances necessary for continued local growth are not made available in physiologic amounts. Results of experiments now in progress in this laboratory concerning the effects of urethane on the estrus cycle of the rat would seem to lead to the same conclusion. Vaginal mitotic activity, stopped by urethane, can be restored to normal by the parenteral administration of the estrogenic growth factor, despite the continued administration of 1,000 mg. of urethane per kilogram daily.

Regression of neoplastic growth has been achieved by nonspecific means which debilitate the animal. For example, the marked inhibitory effect of the carcinogenic hydrocarbons for mouse tumors (*19*) has been shown to be attributable to the great loss of weight that these compounds cause (*20*). In the latter study, of five transplantable tumors, only one, a mouse leukemia, was adversely affected by the carcinogen when the debilitating effect of these substances was adequately controlled. Urethane, however, does not inhibit cellular proliferation by causing starvation. Inanition alone does not inhibit fibroplasia but acts synergistically with an otherwise ineffective amount of urethane to retard markedly fibroblastic and endothelial proliferation. This fact appears to indicate that urethane has a toxic, nonspecific, systemic effect which secondarily affects new cellular growth.

The inability of capillaries to proliferate rapidly enough to aid in healing small, clean, surgical incisions in the presence of toxic doses of urethane may explain the failure of transplanted autogenous cells to grow under similar treatment. The latent period of the growth of a transplanted tumor has been defined as the time necessary for new capillaries to form about the implant. The rapidity of growth after the latent period has been completed is also a measure of the ability of capillaries of the host to form rapidly enough to satisfy the increasing nutritional demands of the transplanted neoplasm (*21*). The prevention by urethane of successful "takes" of transplantable tumors or its retardation of rapidly growing neoplasms may, therefore, be the result of inhibition of capillary proliferation without a primary effect on the malignant cells.

SUMMARY

The effect of urethane on wound healing in the albino rat was studied in order to determine the means by which this substance inhibits cellular proliferation in animals. Histologic study of the wounds of normal and of protein-depleted rats, with and without daily treatment with urethane, showed that (1) in well-tolerated doses, urethane does not inhibit fibroblastic and endothelial proliferation, (2) fibroplasia and capillary formation are inhibited by amounts of urethane causing severe systemic intoxication, and (3) previous depletion of the body protein of the rat enables doses of urethane, which are apparently stimulative in the normal animal, to inhibit fibroblastic and endothelial mitotic activity.

It is concluded that any direct mitotic arresting action that urethane may have is apparently augmented by an unknown systemic metabolic disturbance produced by this drug, and that urethane

does not inhibit cellular proliferation by causing inanition or premature cellular maturation. The inability of capillaries to proliferate in the presence of "mitotic arresting" doses of urethane may be the principal means by which this substance retards neoplastic growth.

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INFLUENCE OF AGE ON THE COPPER AND ZINC CONTENT IN THE EPIDERMIS OF MICE UNDERGOING CARCINOGENESIS WITH METHYLCHOLANTHRENE AND A NOTE ON THE ROLE OF CALCIUM [1] [2]

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INTRODUCTION

In our first paper (*1*), we reported that epidermal cancer induced by methylcholanthrene in mice appeared more quickly and in a higher percentage of young than of old New Buffalo mice, whereas in the young and old CBA strain there was no essential difference in response. Furthermore, a comparison of the two series showed that the young New Buffalo mice developed cancer more quickly and in a higher percentage than did the young CBA mice, while the old mice of both strains reacted to the carcinogen in about the same way. These observations led to the conclusion that strain or hereditary factor was strongest in the young.

Obviously the next step in the study of these age and hereditary factors was to investigate the chemical composition of the epidermises of the different groups of mice to ascertain whether this conditions the response to carcinogens. Because the calcium content of epidermis undergoes significant changes during epidermis carcinogenesis (*2*), the role of this element was investigated first (*3*). We observed in both age groups of the New Buffalo strain that the calcium content of hyperplastic epidermis was decreased nearly 50 percent from the normal although the epidermis of old mice of both strains contained appreciably more calcium than did that of the young. However, the calcium content of hyperplastic epidermis of the old CBA mice was 40 percent less than normal, while that of the young was about 20 percent less. Consequently, these results afforded no explanation for the fact that young New Buffalo mice developed tumors earlier and in a higher percentage of individuals than did the old ones, since there was no significant difference in the calcium drop between the two age groups. In the CBA mice, there was a significant difference between the decrease in the calcium content of the young and old mice, but there was no difference in carcinogenic response.

It had been previously demonstrated that hyperplastic epidermis contains about 50 percent less lipid than normal epidermis (*4*). However, the decrease in total lipid was essentially the same in both age groups of New Buffalo and CBA mice, and, therefore, no relationship appeared to exist between the drop in total lipid and the susceptibility to tumor production (*5*).

In this paper, the content of copper and zinc, which has been found to be markedly altered in epidermal carcinogenesis (*6*), is described.

EXPERIMENTAL PROCEDURE

Swiss and CBA mice of both sexes were used; for each strain there were two age groups: one 3 to 4 months old, and the other 12 to 13 months old. The method of application of the carcinogen and removal of the epidermis from the dermis has already been described (*3*). The samples of epidermis were completely ashed at 450° C. in a muffle furnace, and the copper and zinc contents were determined polarographically (*6; 7, pp. 272, 279*). The amounts of the metals in the epidermis are reported on a wet-weight basis of tissue since the extent of the decrease in the calcium, copper, and zinc contents in hyperplastic epidermis is the same whether nucleoprotein phosphorus or the wet-weight of the tissue is selected as a basis of reference (*3, 8*). Also the decrease in the total lipid is practically the same when the lipid protein nitrogen ratio or the dry weight of the fat-free tissue is employed as a basis of reference (*4, 5*). It would, therefore, appear that the diminution of some of the chemical constituents in hyperplastic mouse epidermis was independent of the basis of reference. All 578 animals used in this study were housed in wooden cages, metal-free on the inside, for several weeks before treatment and throughout the experimental period (*6*).

RESULTS

Inspection of table 1 shows that the epidermal copper content of young Swiss mice is somewhat higher than that of the old mice, whereas the amount of zinc in both is nearly the same. After

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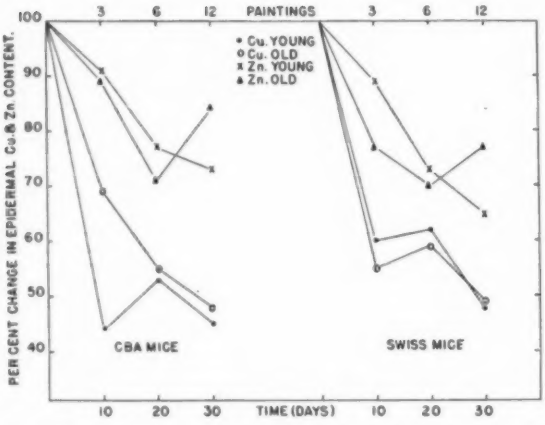

FIGURE 1.—Copper and zinc in epidermal carcinogenesis in young and old Swiss and CBA mice.

3, 6, and 12 applications of the carcinogen, the copper content of the young is lowered, respectively, to 60, 62, and 48 percent of normal while the zinc is decreased, respectively, to 89, 73, and 65 percent. In old mice under similar conditions the copper content is lowered, respectively, to 55, 59, and 49 percent of normal while the zinc is decreased, respectively, to 77, 70, and 77 percent. Therefore, no significant difference with respect to these metals in Swiss mice is apparent because of aging or by reason of application of carcinogen.

The decrease in the content of copper and zinc in hyperplastic epidermis of the young and old CBA mice is nearly the same (table 2) as that which occurred in both age groups of Swiss strain. The hyperplastic epidermis of young CBA mice contains more copper than does that of old, but the amount of zinc is the same.

The results of these experiments are shown graphically in figure 1, where the percentage change of the metal is plotted against the number of applications of carcinogen.

It is apparent that the percentage of change in copper and zinc is nearly identical in the young and old of both strains. The obvious conclusion from this work is that the difference in response of the epidermis of the two strains of mice (young and old) to the action of this carcinogen is not conditioned by the copper and zinc content.

TABLE 1.—*Copper and zinc content of Swiss mice*

NORMAL, UNTREATED MICE

Young mice [1]					Old mice [2]				
Methylcholanthrene paintings (number)	Time after first treatment	Number of mice	Metal per 100 mg. of fresh tissue		Methylcholanthrene paintings	Time after first treatment	Number of mice	Metal per 100 mg. of fresh tissue	
			Copper	Zinc				Copper	Zinc
	Days		*Micrograms*	*Micrograms*	*Number*	*Days*		*Micrograms*	*Micrograms*
		19	0.64	4.9			9	0.70	5.1
		10	.55	5.8			12	.59	4.0
		12	.43	5.5			10	.42	4.9
		18	.50	5.5			10	.50	4.8
		10	.78	4.3			11	.35	4.9
							9	.40	4.7
Total or average		69	.58	5.2			61	.49	4.7

METHYLCHOLANTHRENE-TREATED MICE

3	10	11	0.33	3.8	3	10	8	0.29	3.7
3	10	5	.33	5.3	3	10	12	.31	3.8
3	10	6	.40	4.7	3	10	9	.21	3.4
Total or average		22	.35	4.6			29	.27	3.6
6	20	11	.37	3.6	6	20	12	.32	2.9
6	20	8	.35	3.6	6	20	9	.33	3.5
6	20	9	.36	4.3	6	20	8	.26	3.2
6	20	8	.46	4.3	6	20	5	.24	3.6
6	20	10	.27	3.1					
Total or average		46	.36	3.8			34	.29	3.3
12	30	12	.23	3.4	12	30	10	.27	3.9
12	30	10	.34	3.4	12	30	10	.24	3.4
Total or average		22	.28	3.4			20	.24	3.6

[1] 2-3 months old.

[2] 12-13 months old.

TABLE 2.—*Copper and zinc content of CBA mice*

NORMAL, UNTREATED MICE

Young mice [1]					Old mice [2]				
Methylcholanthrene paintings (number)	Time after first treatment	Number of mice	Metal per 100 mg. of fresh tissue		Methylcholanthrene paintings	Time after first treatment	Number of mice	Metal per 100 mg. of fresh tissue	
			Copper	Zinc				Copper	Zinc
	Days		*Micrograms*	*Micrograms*	*Number*	*Days*		*Micrograms*	*Micrograms*
		10	0.45	5.0			9	0.48	5.0
		10	.70	4.6			10	.38	4.8
		9	.77	6.3			11	.48	5.6
		10	.65	6.0					
Total or average		39	.64	5.5			30	.45	5.1
METHYLCHOLANTHRENE-TREATED MICE									
3	10	7	0.29	4.8	3	10	8	0.42	4.7
3	10	9	.30	6.1	3	10	8	.29	3.9
3	10	8	.18	4.2	3	10	8	.24	4.5
3	10	7	.33	4.8	3	10	9	.21	5.1
Total or average		31	.28	5.0			33	.29	4.5
6	20	13	.43	4.3	6	20	9	.16	2.9
6	20	7	.29	4.2	6	20	8	.25	4.2
6	20	10	.26	5.4	6	20	10	.27	3.8
6	20	12	.38	4.1	6	20			
Total or average		42	.34	4.2			27	.23	3.6
12	30	10	.29	3.9	12	30	10	.20	5.0
12	30	10	.27	3.7	12	30	22	.21	3.6
12	30	10	.36	4.2					
12	30	12	.26	4.2					
Total or average		42	.29	4.0			32	.20	4.3

[1] 2-3 months old.

[2] 12-13 months old.

DISCUSSION

Of the chemical constituents studied in our program on carcinogenesis with respect to the age of the epidermis, there appeared to be no direct relationship between this process and the calcium, total lipid, copper, and zinc contents of epidermis. Only calcium, of the metals investigated, showed a significant difference. In young CBA mice the decrease in calcium in hyperplastic epidermis was much less than in the old group but not proportional to the development of cancers by methylcholanthrene. The epidermis of old mice of both strains contains more calcium than the young, whereas the latter have more copper than the old. The amount of zinc and total lipid is practically the same in all groups.

In our studies on epidermal carcinogenesis during the past 6 years, considerable data have been collected on the content of calcium in this process (*2, 3, 8, 9, 10*), and a recapitulation of this material seems to be desirable at this time. The data are best represented (fig. 2) where the quantity of calcium is shown for the various tissues studied.

The calcium content of the epidermis of Swiss mice rises from 18 mg. in newborn mice to 44 in the adult mouse (2–3 months of age) and to a still higher value (54 mg.) in older mice 12–13 months of age. The values for CBA mice are essentially the same. The epidermis of newborn mice is made up of many layers of cells but is not equipped with hair follicles and sebaceous glands. The epidermal calcium content rises as the structure of the skin approaches the adult condition of thin epidermis, consisting of about two layers of cells plus hair follicles and sebaceous glands. Recent studies in this laboratory have shown that the sebaceous glands and hair follicles are intimately associated with epidermal structure, since treatment with methylcholanthrene reduces the epidermal calcium content to about the same level as that of newborn mice, while the sebaceous glands are destroyed and the epidermis thickens (*5, 11*). Thick, many-layered epidermis of low calcium content without sebaceous glands is found in new-

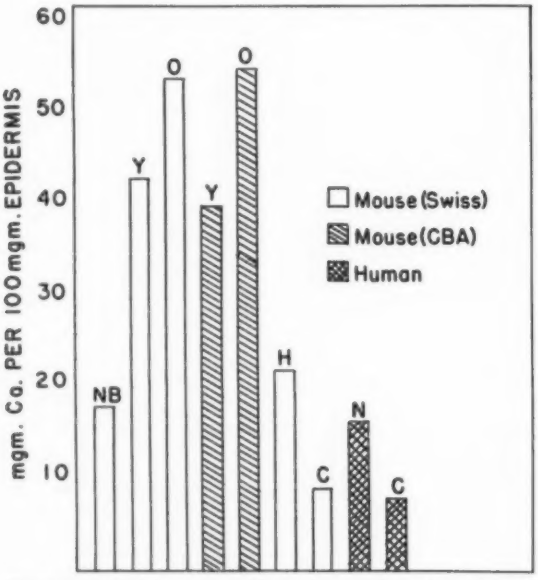

FIGURE 2.—Calcium in epidermal growth and epidermal carcinogenesis. The letters denote the following types of epidermis: NB, newborn; Y, young (2–3 months); O, old (12–13 months); H, hyperplastic; C, carcinoma; N, normal (human).

born mice and in older mice after treatment with this carcinogen.

Normal human epidermis has a calcium content of 16.5 mg. per 100 mg. which is similar to that of the newborn and hyperplastic mouse epidermis. The amounts of other metals in human epidermis, which is a many-layered structure, are also somewhat like that of hyperplastic mouse epidermis (*10*). Our transplantable mouse squamous-cell carcinoma and human carcinomas of the same type have a similar calcium content.

It is quite possible that calcium plays an important role in the genesis of skin cancer, being associated with the mutual adhesiveness of squamous cells (*12*) and, more fundamentally, with permeability (*13*). On the other hand, no direct relationship appears to exist between the calcium content of the epidermis and the response of the young and old mice of the New Buffalo and CBA strains to the carcinogen. The calcium decrease in the epidermis of the young CBA mice was less than in old CBA mice, but there was no difference in the rate and percentage of the tumors formed in response to the carcinogen.

SUMMARY

Epidermal cancer in mice induced by methylcholanthrene appeared more quickly and in a higher percentage of young than of old New Buffalo mice, whereas in the old and young CBA strain there was no essential difference in response. The possible role of copper and zinc as factors in the age difference in the response of the epidermis of the old and young mice of the two strains to the carcinogen was investigated.

The epidermises of the young of both strains were found to contain more copper than the epidermises of older mice, but the amount of zinc was nearly the same in all groups. Methylcholanthrene caused a similar decrease in the copper (50 to 60 percent) and zinc content (average 75 percent of normal) in both the young and old of both strains of mice. Therefore, no relationship was found between the changes in content of these metals in epidermis and the susceptibility of this tissue to tumor formation.

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EFFECTS OF PODOPHYLLIN ON MOUSE SKIN. I. HISTOLOGIC SEQUENCE AFTER A SINGLE DOSE[1,2,3]

By Lester S. King, M. D.

INTRODUCTION

The resin of podophyllin has attracted recent attention because of favorable results obtained with its use on condylomata acuminata. The clinical experience of the author in this regard and notations of relevant literature have been published elsewhere (*1*). In condylomata the pathology of involution has already been described (*2*). King and Sullivan (*3*) reported histologic similarities between the action of podophyllin and colchicine. The latter has been widely used in various types of experimental work; data relevant to oncology were reviewed by Ludford (*4*).

Colchicine has been used by injection, with reliance on hematogenous spread to the tumors under investigation, while podophyllin as a therapeutic agent has from the very beginning been employed as a topical application. In the pathologic studies of King and Sullivan on human material (*2*), certain characteristic enlarged cells with nuclear and mitotic distortions were abundant, but much of the very widespread histologic changes seemed to be nonspecific. The changes were interpreted as evidence of impairment of cellular metabolism produced by the immediate action of the drug. Since observations in treated and regressing tumors were comparable with those seen in normal human skin treated with the drug, experimental study in animals seemed indicated. The present paper is devoted to a description of changes produced in normal mouse skin by a single application of podophyllin and the derived purified product, podophyllotoxin (*5*). Only qualitative observations are given.

MATERIALS AND METHODS

Virgin female mice of the Webster Swiss strain, weighing 17–20 gm., were used.[4] Podophyllin was used as a vaseline ointment of 30-percent strength and also a 20-percent alcoholic solution. In addition, through the courtesy of Dr. W. G. Bywater, of S. B. Penick & Co., a sample of purified podophyllotoxin was obtained, which was used as a 0.5-percent solution in 95 percent alcohol. Application was made with a camel's-hair brush, along the skin of the back.

Observations were made on about 80 animals, which were killed at suitable intervals from 2 hours to 14 days. Formaldehyde fixation and hematoxylin-eosin stains were used.

In approximately half of the animals, a preliminary epilation with barium sulfide paste was made, with an interval of 1–3 days before the application of the podophyllin. Control studies were carried out testing the effects of barium sulfide alone. Minor changes were noted, but after a lapse of 2 days the skin of the epilated mice seemed, on microscopic examination, essentially indistinguishable from normal controls. The specific effects of podophyllin were not influenced by preliminary epilation.

From the qualitative standpoint, no significant difference was observed whether the drug was applied in vaseline or in alcohol solution, and no noteworthy qualitative difference existed between the crude podophyllin or the purified podophyllotoxin.

RESULTS

Early Stage[5]

The normal mouse epidermis was amply described in other publications (*6*). After one application of podophyllin, the earliest pathologic changes were manifested at about 8 hours. The epidermis, although showing its normal two layers of epithelial cells, appeared mildly increased in width, because of slight enlargement in the size of each individual cell. The most striking feature was the presence of numerous distorted mitotic figures, which were single or in small groups or clusters. The chromatin occasionally had the distribution of normal metaphase, forming a tight, compact mass in the center of the well-staining cytoplasm which was only slightly enlarged. More frequently, the chromatin appeared as varying numbers of discrete and separate masses, of variable

[1] Received for publication March 26, 1948.

[2] From the laboratory of the Illinois Masonic Hospital and the Department of Pathology, University of Illinois College of Medicine, Chicago.

[3] Aided by a grant from the National Institute of Health.

[4] Obtained from Rockland Farms, New City, N. Y.

[5] Up to 24 hours after drug application.

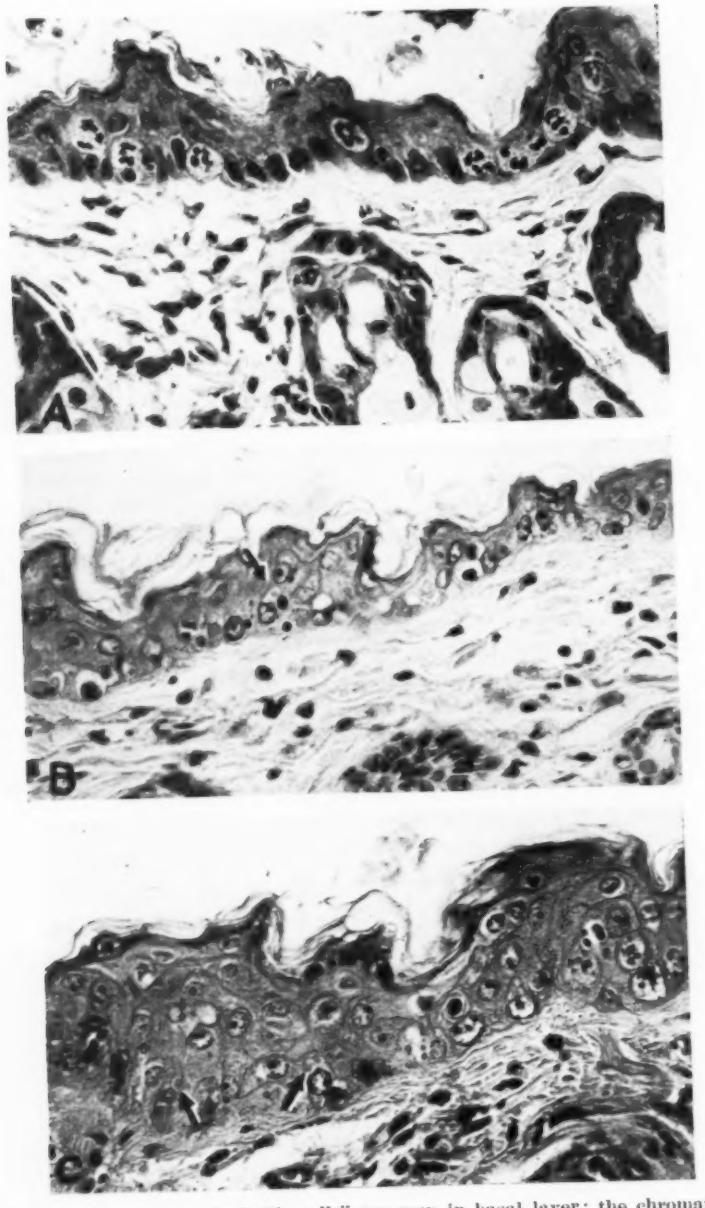

FIGURE 1.—Early stage. *A*, Numerous "podophyllin cells" are seen in basal layer; the chromatin masses vary in size and number, suggesting clumped chromosomes. In the cell in the upper right corner about 24 particles were counted. *B*, In this field, typical podophyllin cells are not observed, but a few pyknotic masses are seen; perinuclear vacuolation is present, and some eosinophilic cytoplasmic inclusions are indicated by arrows. *C*, Thickening of epidermis, due principally to increase in cytoplasmic volume, with slight multiplication in number. Cell membranes are accentuated; inclusions are indicated by arrows; nuclei are moderately enlarged and more vesicular than normal; podophyllin cells are seen; keratohyaline layer is mildly increased. *A*, *B*, and *C*. × 470.

size, but lacking any definite pattern (fig. 1, *A*).[6] Some cells showed as many as 30 individual particles, each of which aparently represented a single chromosome. In other cells, there were 4 to 8 larger, dense, irregular, apparently homogeneous masses of chromatin showing extreme variations in size. These clumps, deeply basophilic, were irregularly dispersed through a bubbly, vacuolated, rarified cytoplasm bounded by a fairly distinct, acidophilic, cytoplasmic membrane. These mitotic disturbances were occasionally seen as early as 3 to 4 hours after drug application but were not present in significant numbers until 8 hours.

The cells in the epidermis that did not show this distorted mitotic change did show some enlargement of the nuclei and a heightened chromatin content. There was also increased prominence of nucleoli. The cytoplasm was generally slightly swollen and enlarged, but cell membranes were not accentuated to any noteworthy degree.

Alterations within 8–12 hours after treatment did not affect the entire epidermis but were intermittent, alternating with short stretches of essentially normal appearing epithelium.

Specimens obtained after an additional interval of 4–8 hours showed a gradual increase in the intensity of these changes. The entire epidermis increased still more in width, and by 16 hours appeared to be at least three cells thick instead of two. In addition, each cell was larger than normal controls. The distorted mitotic figures, called "podophyllin cells" in previous papers, were qualitatively similar to those just described. At 16 hours, noteworthy alterations involved the other nuclei of the epidermis. They were uniformly enlarged to a moderate degree, appeared more vesicular than normal, and exhibited a prominent nucleolus (fig. 1, *B*), or even two to four nucleoli. Cytoplasmic changes were in various forms. One of the most striking was the appearance, within basally placed cells, of small, eosinophilic, cytoplasmic inclusions varying in size from 1μ to 6μ or 7μ, seeming to lie within a surrounding vacuole. Some of these eosinophilic hyaline masses were associated with small, basophilic components. Others remained purely eosinophilic and were surrounded by small, clear halos within the cytoplasm (fig. 1, *B* and *C*).

In general, cytoplasm was moderately enlarged and definitely more vacuolated than normal. Cell membranes were generally accentuated, and intercellular spaces were in sharp contrast with normal mouse epidermis, in which intercellular spaces are lacking.

Keratohyaline granules in the superficial cells of the epidermis were generally accentuated. Two types of granules were seen, one large and relatively coarse, appearing to be an exaggeration of the normal pre-existing keratohyaline, and the other consisting of a delicate, fine dusting of faint blue granules within the increased cytoplasm of the outermost cells. The actual keratin layer was only slightly and inconstantly increased.

At this stage, some deeply eosinophilic, apparently pyknotic cells with dark-staining cytoplasm and small, scattered karyorrhectic nuclear fragments were rarely seen.

After 24 hours of exposure to the drug, these changes were generally more uniformly distributed over the entire painted epidermis. By this time, the keratin material on the surface appeared to be definitely increased. The entire epidermis seemed to be broadened because of increase of both size and number of individual cells. The cell membranes in the surface-lying elements were markedly increased in prominence, while in the more basal-lying cells, the intercellular spaces were usually clear-cut and prominent. The nucleoli also were far more prominent at this stage. The swollen, enlarged cytoplasm was not homogeneous but appeared to be granular, or finely reticulated, or very delicately vacuolated. Numerous cells showed a large perinuclear vacuole, intervening between the nuclear membranes and the cytoplasm.

The significant changes seen during this early period consisted, therefore, of progressive increase in size of the cytoplasm and nuclei, occurrence of abundant podophyllin cells, presence of eosinophilic inclusions, a spongy appearance of the cytoplasm, increased prominence of cell membranes peripherally and of intercellular spaces basally, increase in number of keratohyaline granules, and slight increase in amount of surface keratin.

The chronology herein represents an average of many observations. There was considerable

[6] All photomicrographs were taken at a uniform enlargement of 470 diameters; the relative size of cells may be fairly compared, therefore, by reference to them.

individual variation in animals. One mouse at 8 hours might show more severe changes than another at 12 or 16. Sullivan and King (*1*, *2*) noted different degrees of susceptibility to podophyllin for normal human skin, and mouse skin is apparently subject to similar variations.

It is also probable that even with the exercise of great care the drug was not applied with complete uniformity over the painted area. In addition, variable amounts of the topical application were licked off or rubbed away. Consequently, emphasis is placed on stages rather than on hourly alterations.

Florid Stage

The changes in the florid stage reached a maximum intensity between 24 and 48 hours, and rarely up to 72 hours. The features noted as characteristic of the early stage were seen with regularity

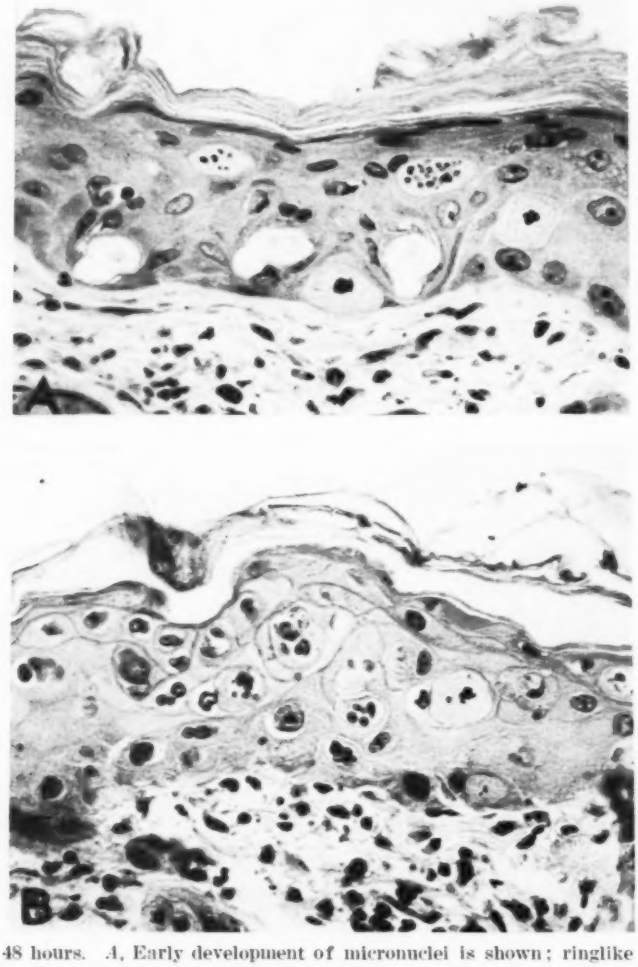

FIGURE 2.—Florid stage, 48 hours. *A*, Early development of micronuclei is shown; ringlike character of some of the chromatin particles can be appreciated, although eosinophilia of central zones is not shown. This cell may be contrasted with other podophyllin cells where the chromatin particles are homogeneous. Two cells have markedly reticulated cytoplasm, and the chromatin material is densely clustered in a tight central mass. Increase of surface keratin is noteworthy. *B*, There is loss of distinction between the superficial and the basal epithelium. In the superficial portion, cell membranes are markedly accentuated, and there is a very faint dusting with keratohyaline granules. Podophyllin cells are numerous but with variable morphology. Cells with micronuclei are clearly seen; one cell with considerably enlarged nucleus and prominent nucleolus is in the center. *A* and *B*. × 470.

and prominence and affected the entire painted surface, so that the distinction between the two phases is largely quantitative. One new aspect, the frequent appearance of so-called micronuclei, was previously noted (*7*, *8*) after the use of colchicine. In podophyllin-treated animals, there was unusual opportunity to trace the development of this phenomenon.

The typical podophyllin cells showed dispersed chromatin masses which might be uniform or variable in size but which were homogeneous in staining reaction and structure. Observations were made which indicated the transformation of such homogeneous particles to more complex bodies. The earliest phase of this change showed a greatly enlarged cell with finely reticulated cytoplasm, within which were numerous small particles, each with a definite internal structure. The particles varied only moderately in size, but each showed a peripheral rim of basophilic material and a central eosinophilic portion. The particles, which suggested altered chromosomes, did not have a definite membrane (fig. 2, *A*).

Other alterations were interpreted as fuller development of micronuclei. There was diminution in the number of separate particles with corresponding increase in the size of each. At the same time, the internal structure became more complex. A definite nuclear membrane appeared, which surrounded discrete basophilic particles and minute eosinophilic bodies. Figure 2, *B*, gives other examples.

Cells containing micronuclei may persist unchanged while the epidermis enters into the reparative stage. Cells with micronuclei were observed unchanged, being desquamated into the keratin layer. On the other hand, some forms suggested a fusion of micronuclei into larger multilobed nuclei in the mid-portion of the epidermis. Micronuclei were seen in significant numbers in specimens in the florid stage. They were once seen as early as 16 hours after drug application. The changes, which formed a rather characteristic picture (fig. 2, *B*), reached their maximum intensity at an average of 48 hours after drug application. The subsequent course might be reparative, or marked degenerative alterations might lead to cell destruction.

Reparative Stage

Phenomena interpreted as reparative were observed on the average about 72 hours after painting. Many of the features of the florid stage still persisted. Enlarged cells with nuclear abnormalities were abundant. The principal differential detail was in the basal layer where the cells were relatively small, with strongly basophilic cytoplasm. The nuclei, although sometimes larger than normal, were dark-staining, lacking the vesic-

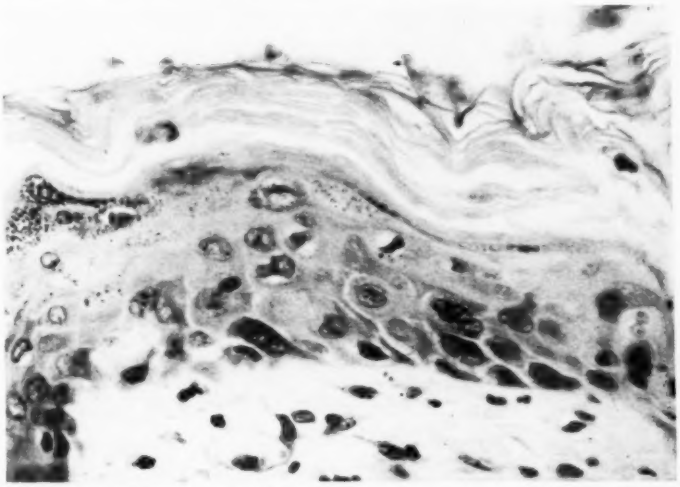

Figure 3.—Early reparative stage, 72 hours. The basal cells are prominent, with large nuclei but scanty basophilic cytoplasm. Intercellular spaces are prominent; multilobulated nucleus may represent fusion of micronuclei. There is an increase in both fine and coarse keratohyaline granules and a marked increase in keratin, with two persisting nuclear masses included therein. × 470.

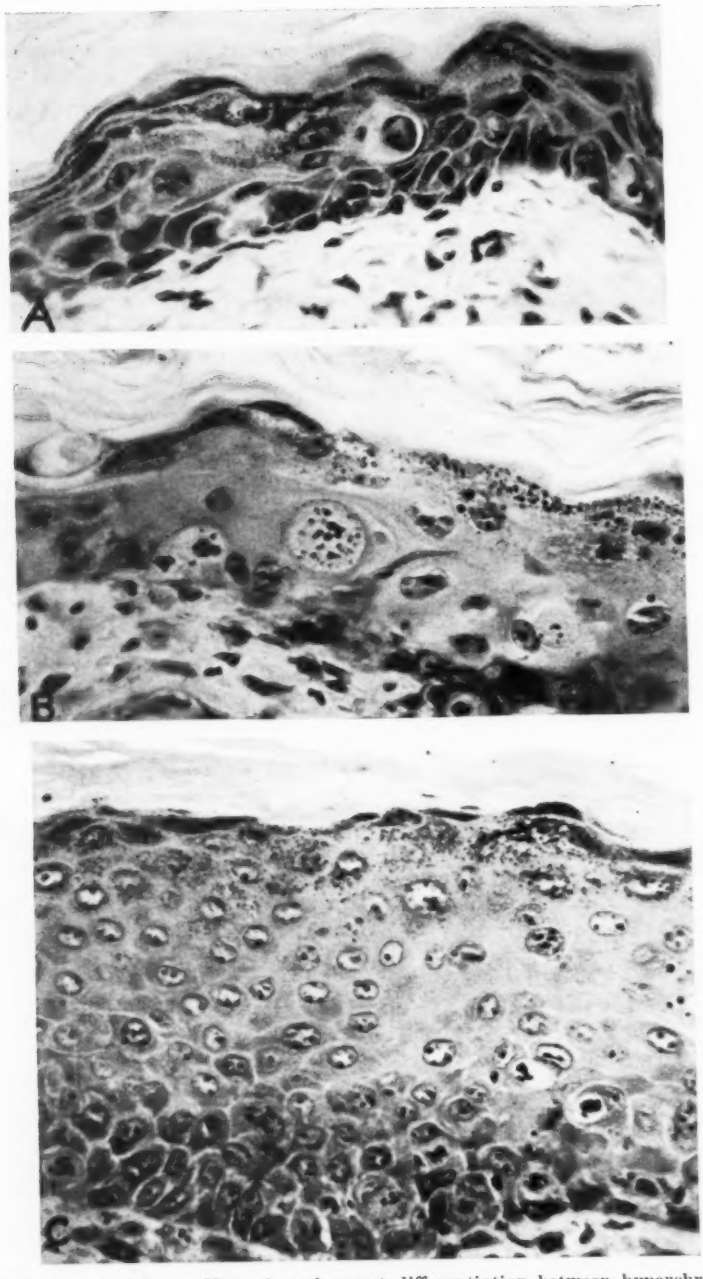

FIGURE 4.—Reparative stage. *A*, 4 days. There is a clear-cut differentiation between hyperchromatic, enlarged, basal cells and the broad granular layer; nuclear forms are still bizarre; one multilobulated nucleus is visible in the lower portion of the figure. In the center is a circumscribed cell, with bright eosinophilic cytoplasm and pyknotic nuclear fragments, which is interpreted as a dead cell being passively carried to the surface by the growth of the basal layer. *B*, 5 days. The prominent podophyllin cell is an example of second wave of activity. (See text.) This cell, probably tetraploid, is much larger than those in figure 1, *A*, which represent the initial effect. The ker-

ular character seen earlier. The smaller amount of cytoplasm gave a greatly increased nucleocytoplasmic ratio (fig. 3, cf. fig. 2, *A* and *B*). Intercellular spaces were still pronounced. The cells in the midportion of the epidermis were more like those in the florid stage. In the uppermost layer, keratohyaline granules were increased in number and prominence; the keratin layer was vastly thicker; and occasionally some parakeratosis was observed.

These phenomena are considered an indication of the reparative stage. A more advanced stage is seen in figure 4, *A* (from a 4-day preparation). The basal layer is thicker, and all the cells of this stratum are small basophilic, with a high nucleocytoplasmic ratio. The keratohyaline layer is also broader and more prominent. The fine granules have become thicker and denser.

The further course of healing was in general a gradual thinning out of the basal and keratohyaline layers. The basophilia diminished, the keratohyaline granules decreased in number and size, and an essentially normal epidermis was reconstituted, with a thickness of two, or, at most, three cells. In different animals this process took place within 6 to 10 days.

Two exceptions were noted in the orderly process of regression and repair. After a single application of podophyllin, the characteristic, enlarged, ballooned cells with dispersed chromatin were seen within the first few days, suggesting an immediate action with gradually diminishing effects. Occasionally, however, even after a single application of the drug, a second wave of podophyllin cells appeared. Figure 4, *B*, shows a portion of skin 5 days after a single drug application. The heavy keratohyaline layer is indicative of healing, and yet in the deeper layers of epidermis two very large podophyllin cells are noted. This repetitive or delayed effect will be discussed subsequently.

Occasionally the healing process following a single application of the drug resulted in a markedly thickened epidermis in which the individual cells showed prominent squamous differentiation, as shown in figure 4, *C*, from a 7-day preparation. The enormous thickening, as compared with figure 1, *A* or *B*, is apparent, as well as the well-marked intercellular spaces, broad keratohyaline layer, and thick mantle of keratin with definite parakeratosis. At the same time, although the drug was applied 7 days previously, the deeper layer showed numerous scattered podophyllin cells. This is a further example of delayed and repetitive action.

Degenerative Stage

After a single application of podophyllin, the epidermis, instead of resolving into an eventually normal pattern, sometimes underwent severe degenerative changes. In such instances, following the florid stage, the cells continued to increase in size. The nuclei also became larger, and the nucleoli, usually one to three in number, became hugely hypertrophic. The nuclear memberane was heavy and the chromatin network dark and compact. The cytoplasm was pale and spongy, with delicate and irregular vacuolations. Cell membranes were prominent. Keratohyaline granules were generally not formed but rarely were seen as minute, isolated dots. There was no distinction between basal and superficial cell layers. The ratio between nucleus and cytoplasm was low, regardless of location of the cell. Cytoplasmic basophilia, characteristic of the regenerative stage, was absent.

At the same time, abundant keratin was formed, without any significant intermediation of keratohyaline granules. Figure 5, *A*, illustrates some of the features of early degenerative processes, taken from an animal killed 4 days after a single dose of podophyllin.

The increase in size sometimes reached extreme and monstrous proportions, as much as 10 times the normal cell size. In the very large cells, the nucleus was correspondingly large, with proportionately larger nucleoli, some of the latter being as large as a normal nucleus. The cytoplasm, which stained palely, was reticulated and spongy (fig. 5, *B*). Not all cells, of course, were of this magnitude.

Although the epidermis appeared considerably thicker than normal, the increased width was not

atin and granular layers are prominent, and the basal layer is less differentiated than in figure 4, *A*. *C*, 7 days. Extreme breadth of epidermis is readily apparent, with clear differentiation of all layers. The specimen resembles human skin in degree of differentiation. Scattered small podophyllin cells seen represent second-wave effect. The massive increase in width of epidermis is unusual after a single application. *A*, *B*, and *C*, × 470.

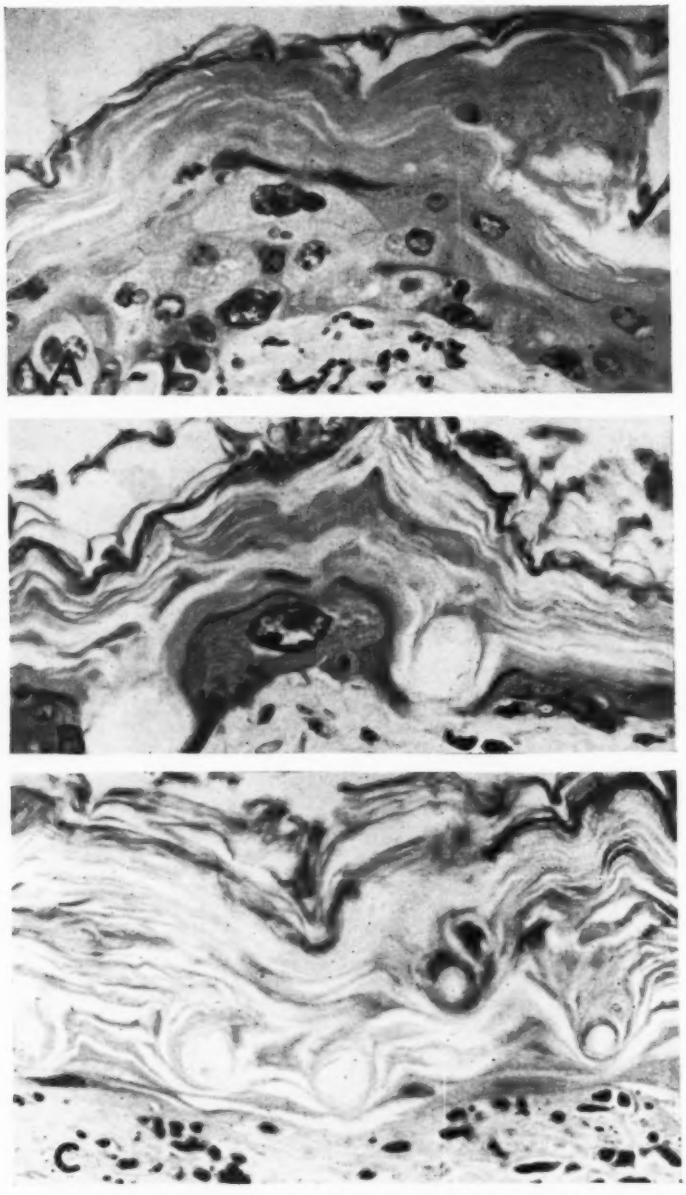

FIGURE 5.—Degenerative stage. *A*, 4 days. The keratin is broad and dense; the epidermal cells are greatly increased in size, with spongy, pale, irregularly vacuolated cytoplasm, large nuclei, and very prominent nucleoli. Loss of differentiation is apparent. Cf. figure 4, *A*. *B*, 5 days. One monster cell intervenes between the dense keratin and the corium. The peculiar fibrillation of the cytoplasm is apparent. Other cells are small, dark, and lacking in differentiation. *C*, 5 days. The epidermis has essentially disappeared, and keratin rests directly on the corium. One undifferentiated and atrophic epithelial cell remains. Circular outlines of hair shafts can be seen. *A*, *B*, and *C*, × 470.

due to multiplication of cells, but in part due to severely increased keratin and in part to increase in size of individual cells. Partial stratification was frequent, but the basally placed elements did not exhibit the usual development of basal cells. Whereas in the reparative phase the basal cells exhibited regenerative activity, in the degenerative stage they remained small, dark, and undifferentiated. The surface cells appeared to be transformed into keratin, without any intermediation of keratohyaline granules.

As the final step in the degeneration of the epidermis, a broad keratin layer rested directly upon the corium. A few crumpled cells still intervened but did not form a continuous layer and did not give evidence of functional activity (fig. 5, *C*). The ultimate fate of such a condition is ulceration, as the keratin sooner or later becomes abraded or desquamates in sheets, exposing the naked corium to bacterial invasion. The sequence of wound healing under the effect of podophyllin has not yet been investigated.

DISCUSSION

The present paper is concerned with qualitative observations in the mouse following a single application of podophyllin. Subsequent papers will deal with multiple applications of the drug, and quantitative data, as well as specific chemical observations.

The initial action of podophyllin appears to be on the basal layer of the epidermis. The podophyllin cell (cf. fig. 1, *A*) is a dramatic and early manifestation of drug action. Such cells, however, are present only in relatively small numbers. These peculiar cytologic changes are not simply arrested mitoses but actual disintegrations of various degrees of intensity. The mechanism responsible for this change has not yet been determined. Some of the distorted cells appear to be reconstituted through the formation of micronuclei. Others die in situ and are passively carried upwards toward the surface by the growth of the epidermis (cf. fig. 4, *A*).

It is clear that the profound cellular changes seen throughout the epidermis are not merely derivatives of the initial nuclear disintegration. Other cells reflect a disturbed metabolism by alterations in nuclear size, cytoplasmic volume, changes in nucleocytoplasmic ratio, nucleolar number and size, cytoplasmic fibrillations, vacuolation, presence of inclusions, and changes in the cell membrane. Changes in Golgi apparatus, mitochondria, glycogen, and lipoid content are in process of study.

Within 24 hours after application of the drug, various alterations were seen, not merely in the basal layer but throughout the broadened epidermis. This sequence suggests that the basal layer, most active metabolically, is most susceptible to drug action, but with the passage of time the other cells react in a more limited fashion.

In the florid stage, the differentiation between basal and superficial cells is no longer clearly marked. This process may be called "de-differentiation," not in the sense of a regression to an earlier primitive cell form but rather a transformation into an indifferent cell type. Normally, the basal layer provides the germinative power, while the superficial layer mediates the conversion into keratin, a process in which keratohyaline granules are apparently concerned. Under the influence of podophyllin, the distinctive features of these layers is at first lost, and all cells exhibit morphologic similarities of characteristic cell type.

In the process of healing or resolution, the basal cells appear dark, with scanty basophilic cytoplasm and chromatin-rich nuclei. At the same time, the superficial cells accumulate kerathohyaline granules. There is thus evidence of resumption of basal-cell regenerative activity and of superficial keratin formation along the usual pattern. The cytoplasmic basophilia in the basal layer, as observed in figures 3 and 4, *A*, may be due to increase of cytoplasmic ribonucleic acid.

On the other hand, when degenerative processes are ascendant, the basally lying cells do not exert a germinative action. The cytoplasm, instead of being scanty and dark, is abundant, pale, and frequently vacuolated. This pallor may possibly be correlated with lack of ribonucleic acid. In the nuclei, the nucleoli assume enormous proportions. The superficial cells appear qualitatively similar to the basal elements. In this sense, the degenerative process can be designated as an increased de-differentiation.

Epidermis, passing through the florid stage and entering the reparative stage, may perhaps be described as undergoing reversible changes. This statement does not imply that all individual altered

cells return to their original normal stage but rather that the epidermis as a unit is not too severely damaged to preclude recovery. Presumably, some cells do return completely to normal while others remain crippled or killed but are passively carried upwards by renewed basal proliferation from below. The occasional second wave of acute podophyllin activity indicates that the drug exerts a long-continued action. There is the implication that the newly proliferating cells are occasionally still susceptible to lingering traces of drug, with the further implication that some adaptation effect has occurred in the majority of cells.

The degenerative stage indicates the presence of irreversible changes. The enormous cells described suggest an inability to divide. The nature of this inhibition and its relation to giant or monster cells in malignant tumors require further study.

A curious phenomenon after podophyllin administration is the formation of keratin in the late stages of epidermal degeneration. The normal formation of keratin is accompanied by abundant keratohyaline granules in the superficial cells. In most pathologic conditions showing hyperkeratosis, there is also increased activity of the basal cells. Yet in degenerating stages in mouse epidermis under the influences of podophyllin, the keratin layer increases markedly in thickness in the absence of both of these phenomena. The evidence suggests that the enlarged and swollen cells are transformed directly into a lamellated, eosinophilic material, which is called keratin. The mode of this transformation is not known. It is possible that the keratin so formed may show distinct qualitative differences from the usual type (or types) of keratin. The solution of this problem requires further histochemical investigation.

Some of the changes observed after podophyllin suggest a kinship to the changes described by Pullinger (*9*) following painting with methylcholanthrene and other carcinogens. These similarities and the possible synergistic effects of methylcholanthrene and podophyllin are being investigated and will be reported in detail in a forthcoming paper.

SUMMARY

The changes produced in mouse epidermis by a single application of podophyllin or podophyllotoxin are described. The early stage (up to 24 hours) was characterized first by the appearance of "podophyllin cells" in the basal layer; then by progressive increases in size of cytoplasm and nuclei; spongy vacuolation of the cytoplasm, with inconstant eosinophilic inclusions; increase in number and prominence of nucleoli; accentuation of cell membranes; appearance of intercellular spaces in the basal layer; and mild increase in amount of keratohyaline granules and of surface keratin.

By 48 hours, the full-blown, or florid picture was apparent, consisting of accentuation and increased prominence of all the changes just listed. In addition, so-called micronuclei were observed, undergoing development from small chromatin particles.

In the florid stage, there was a partial loss of differentiation of cell type within the broadened epidermis.

After 48-72 hours, there might be re-establishment of differentiation leading to repair and healing. This reparative phase was distinguished by small, dark, basal cells, with basophilic cytoplasm and increased keratohyaline in the superficial layer. Bizarre nuclear forms of increased size still persisted, but within 6 to 10 days a normal, or mildly thickened epidermis was generally reconstituted. Occasional exceptions were noted.

Following the florid stage, however, a different sequence might occur, designated as degenerative, in which de-differentiation progressed further, with development of very large, pale cells with enormous nuclei and nucleoli. The end stage of this process was disappearance of the cells and replacement of the entire epidermis by keratin.

The significance of these findings and the problems raised thereby are briefly discussed.

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MAMMARY TUMORS IN MICE PRESUMABLY FREE OF THE MAMMARY-TUMOR AGENT[1]

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INTRODUCTION

In an earlier experiment (*1*), the occurrence of mammary tumors in hybrid mice derived from low-mammary-tumor strain C females and high-mammary-tumor strain C3H males raised the question to what degree the mammary tumor agent is involved in the production of these tumors. A series of investigations to answer this question was begun. This paper deals with one such experiment designed to procure hybrids from inbred strains of mice presumably free of the agent, to ascertain whether they develop mammary tumors, and, if so, whether they exhibit as high an incidence of tumors as those derived from strain C mothers and strain C3H fathers.

In the previous study, most of the tumors were found in mice 18 to 29 months of age; and some were classified as atypical because they did not bear any microscopic resemblance to tumors usually seen in high-mammary-tumor strains of this laboratory but did resemble the mammary-tissue changes known to occur in old female mice. Since the histologic structure of mammary tumors in mice may be related to their etiology, it was considered advisable to describe the histologic types of tumors encountered.

Because strain C mice were known to be very susceptible to the agent (*2*), it was also deemed advisable to use mice from other known susceptible strains as well as from established resistant strains.

Consequently, the object of this report is threefold: (1) To describe briefly the type of tumor usually encountered in mice and to compare this with a second type encountered in old mice: (2) to describe the strains of mice used, including the establishment of two lines of agent-free mice from high-mammary-tumor strains; and (3) to present results of reciprocal breeding between these strains.

MATERIALS AND METHODS

TYPES OF MAMMARY TUMORS

The histology of mammary tumors in mice has been the subject of many studies especially during the early period of work with animal tumors. Although considerable variation exists in the histology of these growths, the majority encountered in the present studies fall into two general types.

The first, designated as type I, reproduce mammary acini and form the typical or basic type of mammary tumor (*3*). They are indistinguishable from tumors occurring in mice known to carry the mammary-tumor agent.

The second, designated as type II, show dilatation of ducts and proliferation of epithelial cells and acini in a relatively abundant stroma (fig. 1).

Haaland (*4*) noted similar histologic structures in the mammary tissue of old female mice and suggested that they may be precancerous because,

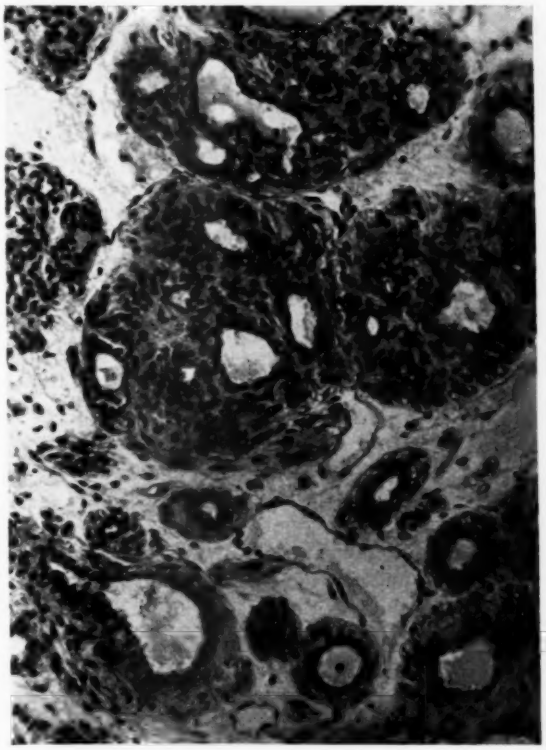

FIGURE 1.—Type II tumor showing dilatation of ducts and epithelial proliferation. May be a precancerous change. Hematoxylin-and-eosin stain. × 200.

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in his experience, mammary tumors appeared to develop within these foci. The chief difference between the growths studied by Haaland and those described here is in size. Haaland designated the condition in his mice as "nodular hypertrophy" and described the nodules as "minute," "small," and "pinpoint," or "just visible to the naked eye," whereas the majority of growths removed from our mice were 12 mm. in diameter; none was less than 6 mm. in diameter. They are called tumors here in the broadest sense of the term because, as Haaland suggested, they may be precancerous and their malignancy has not been established.

The relationship of these type II growths to both the typical (type I) mammary tumor and to the mammary-tumor agent is receiving attention and will be discussed in a later paper of this series.

MOUSE STRAINS

Mice of strains C57 black, I, C, C3H, and dba subline 212 were used. Heston has summarized the development of these strains and their genetic characteristics (*5*). Earlier papers (*2*, *6*) described the establishment of colonies at the National Cancer Institute and carried some information concerning the incidence of mammary tumors. Only brother-to-sister matings have been made since the strains arrived in this laboratory. All have lived under similar environmental conditions and have had the same diet of Purina dog chow and laboratory chow pellets.

A line of C57 black mice was started in 1939 from two litters obtained from Dr. W. S. Murray, of the Springville Biological Station, Springville, N. Y. After 11 generations of inbreeding, selection was made to establish a common female ancestor in the original litters. The colony is now in the F_{21} generation in this laboratory.

A total of 343 breeding females have died at a mean age of 18 months, and of these only 1 developed a mammary tumor. This mouse was 23 months old when her tumor appeared and she was not a descendant of the ancestor of the present colony. Her tumor was classified as type II with an extreme degree of keratinization. An extract of the tumor was administered to appropriate test mice, and none developed a tumor.

These C57 black mice are very resistant to the mammary tumor agent. During the past 3 years, 50 of the females nursed upon strain C3H foster mothers during the entire period lactation and then bore 3 to 6 litters in rapid succession. None developed a mammary tumor. When C57 black mice were suckled by a strain C3H female, they transmitted the agent to their offspring; but none of the offspring transmitted sufficient agent in their milk to produce tumors in suitable test animals (*2*). Apparently the agent disappeared or was inactivated in the C57 black females.

This colony of C57 black mice, in common with others, is a low-mammary-tumor line but may be more resistant to the development of mammary tumors than is the average C57 black colony.

Strain I mice were descendants from a litter procured from the Roscoe B. Jackson Memorial Laboratory in 1935. This litter represented the F_{22} generation of inbreeding. After 10 more generations, selection was made to establish a common female ancestor in the original litter. Further selection was made on the basis of longevity after 20 generations of inbreeding in this laboratory. They are now in the F_{50} generation.

Most strain I mice died or were killed before 1 year of age because of rectal prolapse, but in later generations this tendency decreased, and the animals lived a longer average life span. Approximately 1,550 breeding females that were used to maintain the colony have died and, of these, 1,080 died or were killed before 1 year of age, and the remaining 470 lived to an average age of 15 months. None developed a mammary tumor.

The absence of breast tumors in breeding females, together with the low incidence of such tumors in hybrids derived from strain I females (*7*), established these I mice as a low-mammary-tumor strain.

The mammary-tumor incidence in a colony of strain C mice propagated in this laboratory was reported in another paper (*8*). Over 600 breeding females of the colony lived to an average age of 19 months and exhibited a mammary tumor incidence of less than 1 percent. All tumors thus far have been typical (type I) tumors. These mice are, therefore, a low-mammary-tumor strain.

In conformity with the plan of the experiment, two lines of mammary-agent-free mice were derived from high-mammary-tumor strains C3H and dba.

The line of agent-free C3H mice originated from 2 litters born during May 1942; both consisted of

5 females and 1 male. One was obtained by killing a pregnant mouse and taking the young from her uterus; the other by removing the young from their mother before they ingested any of her milk. Both litters were suckled by strain C57 black females that had given birth to litters 24 hours previously. The fostered females, designated the F generation, were mated to their brothers, and each was permitted to cast 1 litter. The litters contained 28 F_1 generation females.

The F_1 generation mice each bore 1 litter, of which 18 were killed soon after birth, and the remaining 10 were raised. These 10 litters were selected to establish common ancestors in the F generation; 6 were descendants of a mouse obtained by surgical procedure and 4 from a mouse removed from her mother immediately after birth. Two lines were thus established.

Mice of the F_2 and F_3 generations gave birth to one litter only; but starting with the F_4 generation, the colony supplied experimental animals, and each female bore from two to six litters. Selection occurred in the F_8 generation when a common ancestry was chosen on the basis of longevity. This eliminated the line derived from the mouse obtained by post-mortem caesarean because she died when 20 months old, whereas the other F generation mouse lived to the age of 25 months. The colony is now in the F_{14} generation. Beginning with the F_1 generation, all surviving mice were killed at the age of 24 months in order to obtain as many autopsy records as possible and because of space limitations.

Results of 10 completed generations of inbreeding are summarized in table 1. The incidence of mammary tumors was 3 percent at an average age of 22 months. This is in sharp contrast with an incidence of 91 percent at an average age of 8.5 months in the colony of breeding strain C3H females from which the line was derived (*6*). This agent-free line of C3H mice is designated hereafter as C3H−.

TABLE 1.—*Occurrence of mammary tumors in strain C3H breeding females which were free of the mammary-tumor agent*

Generation	Number of mice	Number and type of mammary tumors		Average age at which tumors occurred	Average age at death without tumor
		Type I	Type II		
				Months	*Months*
F	10				23
F_1	28	1		23	23
F_2	23				20
F_3	14				23
F_4	17				22
F_5	27	1		23	23
F_6	23	1		19	21
F_7	50	3	1	22	22
F_8	26				23
F_9	32	1		19	22
F_{10}	26	1		23	21

The F_1 generation mouse (No. 28) in which a mammary tumor arose was a daughter of mouse No. 8, the ancestor of the colony. She reared one daughter which bore but did not raise one litter and died free of tumor when 20 months old. The F_5 generation tumorous mouse (No. 115) belonged to the discontinued line. She gave birth to three litters, but none of her offspring were used to propagate the colony. She had two sister litter mates; both of which died without showing tumor when 24 months old. The F_6 generation animal (No. 146) belonged to the continued line and bore but one litter which did not survive. She did not have a female litter mate, but three sisters from an earlier litter all lived to be 24 months old.

Four mice of the F_7 generation developed mammary tumors. They were 20, 21, 22, and 24 months old when their tumors arose. Two (Nos. 194 and 195) were litter mates. All belonged to the discontinued line, and their progeny were not used to propagate the colony. None had a tumorous mouse in its ancestry. The two (Nos. 151 and 167) from different litters had a total of five female litter mates that lived to an average age of 23 months. Mouse No. 194 developed a hyperplastic (type II) tumor.

The F_9 mouse (No. 258) did not raise her litters. She had one sister litter mate that died at the age of 20 months. The F_{10} mouse (No. 251) also failed to raise her litters and did not have a sister.

Strain dba mice from a colony showing an incidence of 43 percent mammary tumors at an average age of 15 months in breeding females were used to establish a line of agent-free mice. The line was started during 1944 by killing 2 pregnant mice and taking the litters from their uteri. One litter was suckled by a C3H− and the other by a strain I mouse. Each contained 1 female which was bred to her brother when 2 months of age. One raised 4 litters containing 13 females, and the other raised 2 litters containing 7 females. These 20 females comprised the F_1 generation. Selection occurred in the F_4 generation by choosing, as a

common ancestor, the F generation mouse that was suckled by the C3H− female. The colony is now in the F_8 generation. Virtually all females cast from 2 to 5 litters.

Four completed generations contained 96 females that lived to an average age of 20 months and, of these, only 1 developed a mammary tumor. This mouse was one of the F_3 generation and developed a typical (type I) mammary tumor when she was 18 months old. She was not a descendant of the ancestor of the present colony and did not have any descendants in the colony. Three litter mates died free from tumor at an average age of 17 months.

This agent-free line of dba mice is designated hereafter as dba−.

SELECTION OF MALE MICE

In order to avoid as far as possible the presence of the tumor agent in the reciprocal crosses, it was decided to use males which were free of the mammary-tumor agent. This was done because evidence was on hand that the seminal vesicles and their contents contained the agent. This evidence was procured as follows:

Seminal vesicles were removed from strains C or C3H animals known to contain the agent. The C strain males were from the high-mammary-tumor line established by foster nursing upon strain C3H females (*2*). These fostered C mice are hereafter called C+.

Test animals for the activity of the agent were young strain C and (I×C3H)F_1 females (*7*). Each test mouse was mated when 2 months old and bore one litter which was removed from the mother soon after birth. They were then observed for the occurrence of tumors.

EXPERIMENT 1

Seminal vesicles were removed from four 11-month old strain C+ mice, ground with sand, and diluted in 4 cc. of physiologic saline. The mixture was centrifuged at 1,000 r. p. m. for 5 minutes, and 0.1 cc. of the supernatant was injected intraperitoneally into each of six 8- to 16-day old (I×C3H)F_1 females. Four of these mice developed mammary tumors at an average age of 12 months.

EXPERIMENT 2

Seminal vesicles were removed from two 1-year-old strain C+ males and kept at 5° C. for 1½ hours. They were then cut into pieces approximately 2 mm. in length, and 1 piece was injected subcutaneously by means of a 12-gage trocar into each of 15 strain C females, which were 9 to 18 days old. Six developed mammary tumors at an average age of 13 months. Seven litter-mate controls for the injected mice lived to a mean age of 20 months, and none developed a tumor.

EXPERIMENT 3

Seminal vesicles removed from a 7-month-old strain C3H male, were kept at 5° C. for 2½ hours, cut into pieces, and injected into mice as an experiment 2. The recipients were fourteen 12-to-20-day old (I × C3H) F_1 females, of which 11 developed mammary tumors at an average age of 10 months.

The outcome of these three experiments revealed that the seminal vesicles and contents contained the mammary-tumor agent and suggested the possibility that males may transmit the agent to females. This possibility is receiving further consideration.

In the present experiment, the findings were considered sufficient reason to use only males which were presumably free of the agent. Hence, males from the C3H− and dba− lines were used in all the reciprocal matings.

EXPERIMENTAL PROCEDURES AND RESULTS

The experiment consisted of reciprocal matings between mice of strains C57 black, I, C, and the agent-free lines of strains C3H and dba. Most females raised two or three litters, which they bore to males of different strains. After the hybrids were weaned, they were kept until 2 months old and bred to males of the same hybrid derivation. As in the previous experiment (*1*), each hybrid mouse cast three litters in rapid succession since they were kept with males continuously, and the litters were killed soon after birth. When the breeding period was over, they were kept eight in a cage and watched for the occurrence of mammary tumors. A histologic diagnosis was made of each tumor.

The results are summarized in table 2. Of 17 mammary tumors that arose in 359 F_1 hybrids,

TABLE 2.—*Occurrence of mammary tumors in F_1 hybrid mice derived from strains C57 black, I, C, C3H— and dba—*

Strain of mother	Strain of father	Number of mice	Number and type of mammary tumors		Average age at which tumors occurred	Average age at death without tumor
			Type I	Type II		
					Months	*Months*
C	I	26		1	25	23
C	dba—	15	2	1	25	24
C	C57 black	28				25
C57 black	I	36				24
C57 black	C	32		1	28	26
C57 black	dba—	29				25
I	C57 black	31				23
I	dba—	11				24
I	C	18				23
C3H—	I	31		2	23	25
C3H—	dba—	25	1	2	23	26
C3H—	C	20	1	1	21	25
dba—	C	14		2	23	23
dba—	I	9	1		25	23
dba—	C3H—	13		1	21	24
dba—	C57 black	21		1	25	22

16 were in 202 F_1 hybrids derived from C, C3H—, or dba— mothers. Of 177 hybrids born to C, C3H—, and dba— fathers, 12 had tumors; of 182 hybrids born to I or C57 black fathers, 5 became tumorous. Analysis of the data according to reciprocal crosses is shown in table 3.

TABLE 3.—*Distribution of tumors according to reciprocal crosses.*

Parent strains	Number of mice	Number developing tumor
C and I	44	1
C and dba—	29	5
C and C57 black	60	1
C57 black and I	67	0
C57 black and dba—	50	1
I and dba—	20	1
C3H— and I	31	2
C3H— and dba—	38	4
C3H— and C	20	2

Eleven, nine, eight, four, and two tumors arose in mice derived from strain dba—, C, C3H—, I, and C57 black, respectively.

None of the mothers of the hybrids developed a mammary tumor. The number of females used to obtain the hybrids and their average age at death are shown in table 4.

TABLE 4.—*Number and average age at death of mothers of F_1 hybrids*

Strain	Number of mice	Average age at death
		Months
C	19	21
C57 black	15	21
C3H—	18	23
I	18	14
dba—	17	20

Distribution of the tumors in the F_1 hybrids according to the strain of mother is presented in table 5. As mentioned previously, many of the females bore litters to males of different strains. Only one female (strain C No. 11) raised more than one mouse which developed a tumor. The tendency toward tumor development in the hybrids was not limited to the offspring of certain females.

DISCUSSION

Acording to the histories of the inbred strains used in this investigation, they did not possess the mammary-tumor agent. Therefore, the occurrence of mammary tumors in a small number of them and in their hybrids is evidence that the agent may not be essential for the production of all mammary tumors in mice.

None of the hybrid groups showed as high an incidence of tumors as those of the previous cross (*1*) in which 75 (C × C3H) F_1 hybrids revealed an incidence of 60 percent. This higher incidence in the C × C3H mice was not due to their longer life span, since those without mammary cancer died at an average age of 24 months, which, as shown in table 2, was comparable with the average age at death of the hybrids used here.

In the current series of crosses, a special effort was made to avoid the possibility of the hybrids' acquiring the agent from their paternal parent; males were used from lines of strains C3H or dba which were free of the agent. This was done because a review of the literature (*9*) revealed that hybrids derived by mating low-tumor-strain females to high-tumor-strain males often showed a

TABLE 5.—*Distribution of tumors in F_1 hybrids according to mother*

Strain of mother	Mother number	Strain of father	Number of litter in which tumor appeared	Number of mice in litter	Type of mammary tumor		Age of mouse developing tumor	Average age at death without tumor
					I	II		
							Months	*Months*
C	1	I	2	4		1	25	26
C	2	dba−	2	4	1		24	17
C	11	dba−	2	3	1		27	28
C	11	dba−	2	3		1	24	28
C57 black	6	C	4	4		1	28	25
C3H−	6	I	3	6		1	23	27
C3H−	7	dba−	1	1		1	27	
C3H−	8	dba−	1	3		1	23	24
C3H−	10	I	2	3		1	23	26
C3H−	12	dba−	2	4	1		18	27
C3H−	17	C	2	5	1		22	25
C3H−	19	C	3	5		1	20	26
dba−	4	C57 black	5	5		1	25	23
dba−	5	I	3	3	1		25	22
dba−	7	C	3	3		1	21	27
dba−	20	C3H−	4	2		1	21	19
dba−	27	C	1	2		1	25	25

higher tumor incidence than did the strain of their maternal parent. Hence, it was conceivable that transmission of the agent from high-tumor-strain males to low-tumor-strain females accounted for the higher tumor rates in their offspring. This possibility received support from the outcome of experiments presented here which showed that the agent was present in the seminal vesicles and contents. Nevertheless, some tumors arose in the hybrids. Apparently, inherited tendencies together with hormonal stimulation were predominating influences in the origin of these tumors.

This interpretation, however, is not applicable to the tumors in the (C × C3H) F_1 hybrids of the earlier paper (*1*), because they may have resulted from transmission of the agent from C3H males to strain C females. The fact that, in the present experiment, only 4 breast tumors arose in 69 F_1 hybrids derived from strain C females and strains I, dba−, or C57 black males suggests that the milk of strain C females did not carry the agent.

Analysis of the data showed that most of the tumors appeared in hybrid descendants of strains C3H−, dba−, and C, which not only possess a predisposition to the development of mammary tumors but which, in the presence of the mammary tumor agent, are high-tumor strains. Thus, there was a parallelism between an inherited tendency to mammary-tumor development and susceptibility to the mammary-tumor agent. If those strains of inbred mice possessing an inherited predisposition to the development of mammary tumors are also susceptible to the mammary-tumor agent, then the role of the agent in the production of tumors could be that of an accelerator which markedly increases the incidence of tumors within the life spans of the mice.

According to this postulation, the mammary-tumor agent plays a role in the production of breast tumors in mice similar to that of chemical carcinogens in the induction of pulmonary tumors. It is recognized that the strains of mice which are most susceptible to the development of spontaneous pulmonary tumors are also the most susceptible to induced tumors and that carcinogens decrease the latent period for their development as well as increase the number of tumors (*10*).

SUMMARY

Since hybridization of strain C females and strain C3H males resulted in F_1 female offspring that showed an incidence of 60 percent mammary tumors, an experiment was performed to ascertain whether F_1 female progeny derived from other strains of mice presumably free of the agent would show as high an incidence of tumors as did the C×C3H cross.

F_1 hybrids obtained by reciprocal breeding of such strains did not reveal as high an incidence as those procured from the C×C3H cross. Seventeen mammary tumors arose in a total of 359 F_1 hybrids, 16 of them in 202 F_1 hybrids derived from females of strains C, C3H−, and dba−, all of which are relatively susceptible to the agent. Implications of the results are discussed.

A description of the inbred mice used is presented, including the derivation, propagation, and tumor incidences of low-mammary-tumor strains

C57 black, I, and C maintained in this laboratory, as well as the establishment of agent-free lines from high-mammary-tumor strains C3H and dba.

The seminal vesicles and contents of high-mammary-tumor strain mice contained the mammary-tumor agent.

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EFFORTS TO DETECT A MAMMARY-TUMOR AGENT IN STRAIN C MICE[1]

By Howard B. Andervont, *Biologist*, and Thelma B. Dunn, *Pathologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The mammary-tumor agent is of prime importance in the occurrence of mammary tumors in inbred strains of mice and offspring derived from high-mammary-tumor strain females, but its rôle in other mice, especially F_1 hybrids derived from low-mammary-tumor strain females, has not been established. In a previous experiment (*1*), hybrid F_1 mice obtained by mating low-mammary-tumor strain C females to high-mammary-strain C3H males showed a 60-percent incidence of tumors, whereas (I × C3H) F_1 hybrids, which were known to be susceptible to the agent, exhibited an incidence of only 8 percent when suckled by mothers of the (C × C3H) F_1 hybrids. Furthermore, extracts of (C × C3H) F_1 tumors failed to produce mammary tumors when administered to appropriate mice. The evidence suggested but did not prove that breast tumors arose in the hybrids in the absence of a tumor agent, because strain C may harbor a relatively weak agent, and the (C × C3H) F_1 hybrids may be more susceptible than the (I × C3H) F_1 animals. In addition, it was known that strain C could propagate an active agent, because a single exposure to C3H milk transformed strain C into a high-mammary-tumor strain for many generations (*2*).

Two experiments were performed to test for an attenuated agent in strain C. In one, an effort was made to activate or enhance the activity of such an agent by passage through successive generations of strain C3H; in the other, an attempt was made to reveal its presence by removing an inhibitor from extracts of lactating mammary glands.

EXPERIMENTAL ANIMALS

All animals were raised in this laboratory. They were maintained under similar environmental conditions, given a constant supply of tap water, and fed Purina Laboratory Chow pellets.

An earlier paper (*2*) contained a brief account of the origin and tumor incidence of the strain C colony maintained in this laboratory. They are descendants of a litter obtained in 1935 from Dr. George D. Snell, of the Roscoe B. Jackson Memorial Laboratory. They were derived from the Bagg albino stock and are designated as B alb C in the list of inbred strains, published by the Roscoe B. Jackson Memorial Laboratory (*3*). The strain was in the F_{35} generation of inbreeding when it arrived in this laboratory and is now in the F_{65} generation. Table 1 summarizes the occurrence of spontaneous pulmonary and mammary tumors in 26 completed generations of breeding females, most of which raised 3 litters apiece.

The incidence of mammary tumors is less than 1 percent at an average age of 22 months. All 6 tumors[2] were classified as typical (type I) mammary tumors because they reproduced mammary acini and formed the typical or basic type of mammary tumor (*4*). None of the tumorous mice had descendants in the colony. In addition to the C mice used to maintain the colony, many others (approximately 1,000) were used in experiments, and these consistently revealed the same low incidence of breast tumors. According to the mammary-tumor history of the strain, it is presumably free of the mammary-tumor agent.

The incidence of spontaneous pulmonary tumors in breeding strain C females is approximately 23 percent at an average age of 21 months. These tumors usually occur singly and are often large. About 20 percent of the mice necropsied at an average age of 22 months show a generalized enlargement of the lymphoid tissues, which has been diagnosed as either lymphoid hyperplasia or leukemia.

Strain C mice are long-lived, and little difficulty is encountered in propagating the strain. They are excellent breeders and raise litters averaging 8 young; sterility is rare in both sexes. During the first 10 generations, pneumonitis was a common cause of death; but this tendency decreased,

[1] Received for publication April 12, 1948.

[2] In the previous description of the strain, seven mammary tumors were listed (*2*). After further study, it was decided not to include one of these in the present report because the tumor was unusual, and its origin could not be determined.

TABLE 1.—*Occurrence of spontaneous pulmonary and mammary tumors in 26 generations of breeding strain C female mice*

Generation	Number of mice	Average age at death	Pulmonary tumors		Mammary tumors	
			Number of mice	Average age of mice when tumor was observed	Number of mice	Average age when tumor occurred
		Months		*Months*		*Months*
F_{15}	2	21	0			
F_{16}	2	10	1	9		
F_{17}	4	11	1	21		
F_{18}	2	13	0			
F_{19}	6	22	1	26		
F_{20}	12	22	8	22		
F_{21}	19	22	7	22		
F_{22}	15	25	3	27	1	29
F_{23}	10	21	4	23		
F_{24}	23	21	4	21		
F_{25}	16	17	3	15		
F_{26}	38	18	11	17		
F_{27}	101	17	14	20		
F_{28}	58	16	15	21	3	21
F_{29}	49	19	7	22		
F_{30}	55	19	12	21	1	20
F_{31}	44	21	16	23		
F_{32}	24	22	4	22		
F_{33}	15	19	6	21	1	18
F_{34}	23	20	6	21		
F_{35}	26	19	6	23		
F_{36}	27	21	8	22		
F_{37}	19	23	4	23		
F_{38}	19	20	7	22		
F_{39}	15	20	2	22		
F_{40}	26	20	7	22		
Totals	650		157		6	

and in more recent generations it has disappeared.

The mice chosen as most appropriate for detecting an agent in strain C milk were from a line of agent-free strain C3H mice established in this laboratory (*4*). In 10 completed generations, their mammary-tumor incidence was 3 percent at an average age of 22 months. The line was derived from a colony of strain C3H mice, which, on the basis of mammary-tumor history, was very susceptible to the mammary-tumor agent (*5*). This line of agent-free C3H mice is herein designated as C3H−.

EXPERIMENTAL PROCEDURE AND RESULTS

Serial-Passage Experiment

A serial-passage experiment was started by transferring 12 C3H− females and their male litter mates, within 17 hours after birth, to 8 strain C foster mothers. The foster mothers represented 6 litters. The fostered C3H− animals comprised the F generation and were the only mice which obtained milk from or had any contact with strain C mice. They were from 11 litters of the F_5 and F_6 generations of the C3H− line and were suckled by F_{55} and F_{56} generation strain C females. Their mothers died free of mammary tumor at an average age of 22 months, and their foster mothers at a mean age of 20 months.

The F generation females were bred to brothers, and 3 subsequent generations were produced by brother-to-sister matings. Each F generation mouse bore 2 litters, of which the second was raised to provide 40 F_1 generation females. Every F_1 generation animal gave birth to 1 litter: 18 litters were killed shortly after birth, and the remaining 22 were raised to supply 59 F_2 generation females. Forty-two mice of the F_2 generation bore 1 litter only which they did not raise; the remaining 17 cast 2 litters apiece; 19 litters were reared to furnish 33 F_3 generation females. Each of 15 females of the F_3 generation had 3 litters, and each of 18 had 2 litters. Most of these litters were raised but were not used in the experiment. When the breeding period was over, the animals were kept 8 in a cage and were observed for the occurrence of mammary tumors.

The results are summarized in table 2. Since the experiment was designed to test for the activity of an agent in the milk of strain C, the test animals were divided into 8 series (A-H) according to the strain C foster mother for the F generation. Of

TABLE 2.—*Occurrence of mammary tumors in fostered C3H— mice and in 3 generations of their descendants*

Generation of fostered C3H— mice	Series A to H, according to strain C foster mother for F generation											
	A			B			C			D		
	Number of mice	Number developing tumor	Average age at death without tumor	Number of mice	Number developing tumor	Average age at death without tumor	Number of mice	Number developing tumor	Average age at death without tumor	Number of mice	Number developing tumor	Average age at death without tumor
			Months			*Months*			*Months*			*Months*
F	1	0	24	1	1		1	0	24	1	0	20
F_1	5	0	27	3	0	26	3	0	21	4	0	20
F_2	7	0	23	9	0	23	5	0	23	8	0	24
F_3	7	0	20	1	0	21	0			2	0	24
Total	20	0		14	1		9	0		15	0	

Generation of fostered C3H— mice	E			F			G			H		
	Number of mice	Number developing tumor	Average age at death without tumor	Number of mice	Number developing tumor	Average age at death without tumor	Number of mice	Number developing tumor	Average age at death without tumor	Number of mice	Number developing tumor	Average age at death without tumor
			Months			*Months*			*Months*			*Months*
F	2	0	16	3	0	22	2	0	23	1	0	30
F_1	3	0	29	10	2	23	7	0	24	5	1	28
F_2	2	0	21	8	0	23	8	0	23	12	0	24
F_3	6	2	25	1	0	24	10	0	21	6	0	17
Total	13	2		27	2		27	0		24	1	

144 mice used in the experiment, 6, or 4 percent, developed mammary tumor, an incidence similar to that of 3 percent in 10 completed generations of the C3H— line (*4*).

None of the mice of series A, C, D, or G developed a breast tumor. The series B animal belonged to the fostered F generation and was 21 months old when its tumor appeared. The tumor was classified as a typical mammary tumor. One gram of tumor was ground with sand and diluted in 4 cc. of physiologic saline, and 0.1 cc. of the suspension was fed, by means of a stomach tube, to seven (I×C3H) F_1 hybrids, which were 10 days of age. All died free of tumor at an average age of 22 months.

Both tumorous series E mice were in the F_3 generation, and both reared 3 litters, the females of which died nontumorous. The series E mice were litter mates and the only females born to their mother; she died when 20 months old. Both developed typical mammary tumors, one at the age of 24 and the other at the age of 20 months. A portion of 1 tumor weighing 0.7 gm. was ground with sand, diluted in 5 cc. of distilled water, and 0.1 to 0.4 cc. of the extract was injected intraperitoneally into 12 strain C mice, which were 6 to 30 days old. The larger dose was given to the older animals. All died free of tumor at an average age of 18 months.

The two tumorous F_1 generation mice of series F were also litter mates; a third female litter mate died when 9 months old. Their mother lived to be 18 months old. One developed a tumor at the age of 20 months. She raised one litter containing five females, each of which gave birth to one litter, and died free of tumor at a mean age of 26 months. Her tumor was diagnosed as atypical and was histologically the type II tumor previously described, characterized by dilatation ducts and epithelial proliferation in an abundant, delicate stroma, with areas of lymphocytic infiltration (*4*). One gram of tumor tissue was ground with sand, diluted in 4 cc. of physiologic saline, and 0.1 cc. of the extract was injected intraperitoneally into nine 9-day-old C females. All died tumor-free at an average age of 22 months.

The other series F mouse became tumorous when 25 months old. She raised one litter containing two females, each of which cast two litters. Both died tumor-free, one at the age of 23 months and

the other at 27 months. Her tumor was a typical mammary tumor. One gram of tissue was ground with sand, diluted in 5 cc. of distilled water, centrifuged at 2,000 r. p. m. for 15 minutes, and 0.4 cc. of the supernatant was injected into each of four (I×C3H) F_1 hybrids and five strain C females, all of which were 1 month old. All lived to the age of 21 months, and none developed a breast tumor.

A tumor arose in the series H mouse of the F_1 generation when she was 28 months old. Her tumor was diagnosed as atypical (type II) because it appeared to have developed mainly from the ducts; considerable keratinization was also present; there were areas of calcification and ossification in the stroma and evidence of inflammation with leukocytic infiltration. She raised one litter containing three females, each of which had three litters, and all died free of tumor at the average age of 20 months.

In order to ascertain whether the C3H− mice used in the experiment were susceptible to the agent, female offspring born to F_3 generation females were removed from their mothers within 17 hours after birth and were suckled by strain C3H females from the colony. They remained with their foster mothers for 1 month, were bred at the age of 2 months, and gave birth to 1 litter, which was killed soon after birth. Of 15 mice, all developed mammary tumor at an average age of 11 months. Apparently the low incidence of breast tumors in the C3H− mice of the experiment was not attributable to their resistance to the agent.

Adsorption Experiment

An adsorption experiment was performed to ascertain whether milk of strain C animals contained an inhibitor, which masked the activity of the agent. Previous studies, which are described later, had suggested the presence of an inhibitor in extracts of mammary tumors or lactating glands.

All experiments were performed at room temperature. The test mice were mated when 2 or 3 months old and were permitted to bear one litter, which was removed from their mothers soon after birth. They were then kept eight to a cage and were observed for the occurrence of breast tumors.

Experiment 1[4]

A mammary tumor was removed from a strain A female; 1.8 gm. was ground with glass, suspended in 20 cc. of distilled water, and centrifuged at 1,500 r. p. m. for 15 minutes. The supernatant, which was pipetted off, was called the tumor extract. One gram of finely ground montmorillonite was added to 5 cc. of the tumor extract and stirred for 5 minutes. The mixture was then centrifuged at 3,500 r. p. m. for 15 minutes, and the clear supernatant poured off. This was called the montmorillonite supernatant. The sediment was resuspended in 5 cc. of distilled water and called the montmorillonite sediment.

The tumor extract, montmorillonite supernatant, and montmorillonite sediment were injected into (I × C3H)F_1 hydrids which were 12 to 29 days old; each received 0.2 cc. intraperitoneally. Litter mates served as test animals for each substance. Of 8 receiving the tumor extract, 1 developed a tumor when 15 months old, and the remaining 7 lived to a mean age of 25 months. Of 10 receiving the montmorillonite supernatant, all developed tumors at an average age of 10 months. Of 6 receiving the montmorillonite sediment, 4 developed tumors at an average age of 13 months, and 2 died when 26 months old.

Experiment 2

A mammary tumor was removed from a strain C3H female. One gram was minced in a mincing machine, ground with sand, and diluted in 20 cc. of distilled water. The suspension was centrifuged at 1,500 r. p. m. for 15 minutes, and the supernatant was pipetted off. One gram of montmorillonite was added to 5 cc. of the supernatant and mixed thoroughly for 5 minutes. The mixture was then centrifuged at 3,500 r. p. m. for 20 minutes, and the clear supernatant was poured off. The sediment was resuspended in 5 cc. of distilled water. The tumor extract, montmorillonite supernatant, and montmorillonite sediment were injected into strain C mice which were 4 to 18 days old. Each mouse received 0.1 cc. intraperitoneally. Litter mates were used as test animals for each substance. Of 10 receiving the tumor extract, 5 developed tumors at an average age of 14 months; the

[4] This experiment was conducted in collaboration with A. H. Hanszen (6), who was studying the adsorbent properties of montmorillonite at the National Cancer Institute.

survivors lived to an average age of 19 months. Of 13 receiving the montmorillonite supernatant, 10 developed tumors at an average of 10 months; the 3 nontumorous mice died at an average age of 17 months. Of 10 receiving the montmorillonite sediment 7 developed tumors at an average age of 11 months; the 3 remaining mice died at a mean age of 14 months.

The results of experiments 1 and 2 are summarized in table 3.

TABLE 3.—*Test of adsorbent properties of montmorillonite on extracts of mammary tumors*

Inoculum	Number of mice	Number developing tumor	Average age when tumors appeared
			Months
Tumor extract	18	6	14
Montmorillonite supernatant	23	20	10
Montmorillonite sediment	16	11	12

EXPERIMENT 3

Lactating mammary glands were removed from a strain C3H female that had given birth to her litter 14 days previously and had been removed from her litter for 17 hours. The engorged glands were passed through a fine mincing machine and strained through fine gauze. This material was diluted by adding 9 cc. of distilled water to each cubic centimeter, and the resultant mixture was centrifuged at 2,500 r. p. m., for 15 minutes. The supernatant was pipetted off and called mammary-gland extract. One gram of montmorillonite was added to 5 cc. of the extract and stirred for 5 minutes. This mixture and 5 cc. of mammary-gland extract were centrifuged at 2,000 r. p. m. for 20 minutes. The opalescent supernatant from the montmorillonite mixture was poured off and called montmorillonite supernatant 1:10. The supernatant from the mammary-gland extract was called mammary-gland extract 1:10. The montmorillonite sediment was resuspended in 5 cc. of distilled water and called montmorillonite sediment. The mammary-gland extract 1:10 and the montmorillonite supernatant 1:10 were each used to make a tenfold dilution. Thus, five substances were obtained for injection into 7- to 21-day-old strain C females; each received 0.1 cc. intraperitoneally. Litter mates were used as test animals for each material. The results are sumarized in table 4.

TABLE 4.—*Test of adsorbent properties of montmorillonite on an extract of lactating mammary glands*

Inoculum	Number of mice	Number developing tumor	Average age when tumors appeared	Average age of mice dying without tumor
			Months	*Months*
Mammary gland extract 1:10	10	4	15	19
Montmorillonite supernatant 1:10	10	9	12	19
Mammary gland extract 1:100	10	5	15	22
Montmorillonite supernatant 1:100	10	10	11	.....
Montmorillonite sediment	6	1	15	20

The results of these three experiments suggested that the montmorillonite removed an inhibitor from the extracts of tumor or lactating mammary glands.

Several experiments were performed in which extracts of lactating mammary-glands from strain C mice were treated as in experiment 3. Details of the experiments are not presented because none of the injected animals developed a tumor. If the lactating mammary glands of strain C contained an agent, the procedure failed to reveal its presence.

DISCUSSION

The inability of strain C milk to increase the incidence of mammary tumors in C3H− mice indicates that the milk either did not contain an agent, or, if present, its activity was not enhanced by passage through three generations of C3H− animals. Failure to detect or activate an agent in the milk of strain C suggests that the relatively high incidence of mammary tumors in the (C × C3H) F_1 hybrids of the early experiment (*1*) was not due to an agent in their mothers' milk. However, the findings are not interpreted as showing that a mammary-tumor agent was not involved in the occurrence of tumors in the (C × C3H)F_1 hybrids for the following reasons:

(1) Introduction of strain C milk into C3H− mice may not be the proper procedure for activating a relatively weak agent in strain C milk.

(2) The agent was found in the seminal vesicles and contents of strain C3H males (*4*). Attentuation of the C3H agent when transmitted from C3H males to C females could be responsible for the tumors in their offspring.

The unsuccessful attempts to reveal an agent in extracts of lactating mammary glands by an adsorption technique require little discussion.

Experimental evidence that montmorillonite adsorbed an inhibitor from mammary-tumor extracts, as well as from extracts of lactating mammary glands containing the agent, suggested that such an inhibitor may mask the activity of a weak agent in strain C mice. Under the conditions of these experiments this was not the case.

SUMMARY

The derivation and tumor incidence of low-mammary-tumor-strain C mice is given. Breeding females showed a mammary-tumor incidence of less than 1 percent at an average age of 22 months and a pulmonary-tumor incidence of 23 percent at an average age of 21 months.

Since (C × C3H)F_1 hybrids showed a mammary-tumor incidence of 60 percent, the milk and lactating mammary glands of strain C were tested for the presence of a mammary-tumor agent.

Eight strain C females suckled twelve strain C3H mice from a mammary-tumor-agent-free line. The fostered mice and 3 generations of descendants were observed for the occurrence of mammary tumors. Of 144 mice, only 6 developed mammary tumors. Strain C milk failed to increase the incidence of tumors in the mammary-tumor-agent-free C3H line.

Experiments indicated that the adsorbent, montmorillonite, removed an inhibitor from extracts of mammary tumors or lactating mammary glands containing the mammary-tumor agent. Exposure of strain C milk to the adsorbent failed to reveal the presence of a mammary-tumor agent in the milk.

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ABSTRACTS

INTERFERENCE WITH ESTROGEN INDUCED TISSUE GROWTH IN THE CHICK GENITAL TRACT BY A FOLIC-ACID ANTAGONIST, by *Roy Hertz.* Science 107 : 200 (1948)

The feeding of a folic-acid antagonist markedly reduces the tissue-growth response to stilbestrol in the female-chick genital tract. This inhibition is completely reversible by excess folic acid. The potential usefulness of this type of interference with hormonal action in the case of hormone-dependent tumors is discussed.

DIETARY IMPAIRMENT OF ESTROGEN RESPONSE IN THE IMMATURE MONKEY, by *Roy Hertz.* Proc. soc. Exper. Biol. & Med. 67 : 113–115 (1948)

Of eight sexually immature monkeys maintained on a folic-acid-deficient, synthetic diet until evidence of dietary deficiency appeared, six failed to show the characteristic response to estrogen administration seen in normal monkeys. Two control monkeys, fed the same diet plus liver, and two of the apparently deficient animals showed typical estrogen response to similar hormone treatment.

FURTHER STUDIES ON α,α—DI(ACYLAMINO) PROPIONIC ACIDS, by *Vincent E. Price, Maurice Errera,* and *Jesse P. Greenstein.* Arch. Biochem. 17 : 51–60 (1948).

α,α—Di(*dl*-chloropropionylamino)propionic acid was prepared by condensing two moles of *dl*-chloropropionitrile with one mole of pyruvic acid in chilled, concentrated sulfuric acid. On partial amination, α—(*dl*-alanylamino)-α—(*dl*-chloropropionylamino)propionic acid was obtained, which on further amination yielded α,α—di(*dl*-alanylamino) propionic acid · HCl.

The susceptibility to enzymatic hydrolisis of the three compounds was determined in rat kidney digests by the ammonia and pyruvic acid produced. Di(chloropropionylamino)propionic acid was completely resistant. (Alanylamino)(chloroproprioylamino)propionic acid yielded a maximum of 0.5 moles of ammonia N and of 0.5 moles of pyruvic acid per mole substrate. At each time interval during the incubation period the molar ratio of these products was close to unity. Di(alanylamino) propionic acid yielded ammonia N and pyruvic acid in digests with rat kidney at a nearly constant molar ratio of 1.6.

The optically isomeric forms of the substrates are described and are considered in the interpretation of the phenomena observed.

EFFECT OF PHOSPHATE AND OTHER ANIONS ON THE ENZYMATIC DESAMIDATION OF VARIOUS AMIDES, by *Jesse P. Greenstein* and *Florence M. Leuthardt.* Arch. Biochem. 17 : 105–114 (1948).

The effect was studied of added phosphate, arsenate, sulfate, nitrate, ethylphosphonate, methylarsonate, benzenesulfonate, pyruvate, and pyruvoylglycinate on the desamidation of glutamine in aqueous extracts of various rat tissues. Phosphate, arsenate, sulfate, and ethylphosphonate accelerated the desamidation of the substrate in extracts of liver, brain, and spleen, but not in extracts of kidney. Pyruvate accelerated the desamidation only in liver. The optimum pH of 8.6 was obtained in digests of glutamine with brain extracts with or without added phosphate. The maximum effective concentration of phosphate was reached at a ratio of about 2 moles of phosphate per mole of glutamine.

Phosphate, arsenate, and sulfate had no effect on the desamidation of asparagine, isoglutamine, glycine amide, alanine amide, or leucine amide. Phosphate and arsenate produced a marked inhibition of the capacity of tissue extracts to hydrolyze benzoylarginineamide, the optimum pH for benzoylargininеamidase activity, 6.2, being the same in the presence and absence of added phosphate.

An explanation for the phenomena noted is advanced, based upon the possibility of a 2-step reaction involving a combination of the anion with the substrate, followed by the hydrolysis of the labile intermediate compound.

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